Exhibit B

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Effect of Time of Insemination on Number of Accessory Sperm, Fertilization Rate, and Embryo Quality in Nonlactating Dairy Cattle

J. C. Dalton,[1] S. Nadir, J. H. Bame, M. Noftsinger,
R. L. Nebel, and R. G. Saacke
Department of Dairy Science
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061

ABSTRACT

Two experiments were conducted to determine the effect of insemination time on number of accessory sperm per embryo (ovum), fertilization rate, and embryo quality. Semen was collected from three fertile Holstein bulls and cryopreserved in egg yolk-citrate-glycerol. In experiment 1, cows were continuously monitored for behavioral estrus by the HeatWatch estrous detection system and were artificially inseminated (AI) with one 0.5-ml straw (25×10^6 sperm) at the onset of estrus (AI 0 h), 12 h after onset (AI 12 h), or received natural service at 0 h (Nat 0 h) from one of three bulls. From 150 inseminations, 115 embryos and ova (AI 0 h: n = 39; AI 12 h: n = 39; Nat 0 h: n = 37) were recovered 6 or 7 d after insemination. Fertilization rates differed between treatments (AI 0 h: 67%; AI 12 h: 79%; Nat 0 h: 98%). Median accessory sperm per embryo (ovum) also differed (AI 0 h: 1; AI 12 h: 10; and Nat 0 h: 27) and paralleled the fertilization rate. Embryo quality was not affected by insemination time or natural service. In experiment 2, cows received AI at 0, 12, or 24 h (AI 24 h) after the onset of estrus as determined by HeatWatch. From 154 inseminations, 117 embryos and ova (AI 0 h: n = 39; AI 12 h: n = 39; AI 24 h: n = 39) were recovered 6 or 7 d after insemination. Fertilization rates did not differ in experiment 2 (AI 0 h: 66%; AI 12 h: 74%; AI 24 h: 82%); however, a trend toward a higher fertilization rate accompanied AI 24 h. Median accessory sperm values increased from AI 0 h (1) to AI 24 h (4). Embryo quality declined with AI at increasing intervals after onset of estrus, as percentages of excellent and good, fair and poor, and degenerate embryos were as follows: 77, 15, 8; 52, 38, 10; and 47, 19, 34 for the 0-, 12-, and 24-h inseminations, respectively. Results indicate AI 12 h after the onset of estrus provides a compromise between potential fertilization failure (AI 0 h) and embryo failure (AI 24 h), despite increased accessory sperm per embryo (ovum) after AI 24 h. Artificial insemination 12 h after onset of estrus should optimize fertility of dairy cattle through an acceptable fertilization rate, number of accessory sperm per embryo, and desirable embryo quality.
(**Key words:** artificial insemination, accessory sperm, embryo quality, dairy cattle)

Abbreviation key: Nat 0 h = natural service 0 h after onset of estrus.

INTRODUCTION

The US dairy industry loses greater than $300 million annually as a result of reproductive inefficiency, primarily due to the failure to detect estrus, the misdiagnosis of estrus, or inappropriate timing of AI (Senger, 1994). Heat detection efficiency is usually less than 50% in most herds, and heat detection accuracy varies widely because up to 5% of all cows presented for AI have been reported as not in estrus based on high milk progesterone levels (Reimers et al., 1985). Therefore, the efficient and accurate detection of estrus and the timing of AI remain current challenges in dairy production. In synchronization of estrus and controlled ovulation schemes, clear guidelines also must be established to optimize insemination time with respect to ovulation.

Numerous studies have investigated the optimal time of AI relative to the stage of estrus. Trimberger and Davis (1943) and Trimberger (1948) reported maximum conception rates in dairy cattle were realized after AI during midestrus until a few hours after the end of behavioral estrus. This work led to the development of the a.m.–p.m. guideline and assumed an intensive, visual estrous detection regimen. In contrast, Foote (1978) and Nebel et al. (1994) reported similar nonreturn rates for once daily AI and AI following the a.m.–p.m. guideline under farm conditions where less intense estrus detection was practiced. By using the HeatWatch estrus detection system (DDx Inc., Denver, CO), Dransfield et al. (1998) determined that the highest concep-

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Corresponding author: J. C. Dalton; e-mail: jdalton@uidaho.edu.
[1]Present address: Caldwell Research and Extension Center, University of Idaho, 16952 South 10th Avenue, Caldwell, ID 83607.

tion rates in 17 commercial dairy herds resulted from AI between 4 to 16 h after the onset of estrus as determined by the first standing event.

Accessory sperm trapped in the zona pellucida by the zona reaction after penetration of the fertilizing spermatozoon are believed to reflect the number of spermatozoa competing for fertilization in cattle (Saacke et al., 1998). Also, the accessory sperm number per embryo (ovum) has been positively associated with fertilization rate (DeJarnette et al., 1992; Nadir et al., 1993; Wilmut and Hunter, 1984) and embryo quality (DeJarnette et al., 1992; Nadir et al., 1993) in single-ovulating cattle (review; Saacke et al., 2000).

Although previous studies on time of insemination provide insight into the optimal time of AI (Dransfield et al., 1998; Trimberger 1948; Trimberger and Davis, 1943), those studies do not describe the relationship between insemination time and fertilization status, sperm accessibility to the ovum as measured by accessory sperm number per embryo (ovum), or embryo quality. Each of these factors is important to establishing pregnancy. The degree to which insemination time affects these factors may lead to techniques that will enhance reproductive success in dairy cattle. Taken together with the realization that estrus detection is the primary problem limiting reproduction of dairy cattle, the present studies used the HeatWatch estrus detection system to more precisely determine time of insemination relative to onset of estrus. The objectives of these experiments were to determine the effect of natural service and time of AI on fertilization rate, embryo quality, and accessory sperm number in dairy cattle.

MATERIALS AND METHODS

Animals and Semen

Experiment 1 was conducted between October 30, 1995, and October 1, 1996 (48 wk). Experiment 2 was conducted between October 1, 1996, and August 4, 1997 (44 wk). All animal experimentation was undertaken by guidelines set forth by the animal use and care committee for Virginia Polytechnic Institute and State University. Each week during the study, five to seven nonlactating Holstein cows, 2 to 10 yr of age, were selected at random for estrus synchronization from a variably sized research herd averaging 30 cows. Estrus was induced with $PGF_{2\alpha}$ (25 mg i.m., Lutalyse; Pharmacia Animal Health Co., Kalamazoo, MI). All injections were performed in the gluteal muscle, and cows were immediately returned to the pasture and herd for estrus detection. Based on rectal palpation, all experimental cows had no abnormalities of the reproductive tract. Before use in either experiment, all cows exhibited estrous cycles between 18 and 24 d in duration.

Three Holstein bulls, ranging from 2 to 9 yr of age, were selected for their ability to produce neat semen with characteristics equal to or greater than 70% morphologically normal sperm and 60% estimated progressive motility (before cryopreservation). Three times yearly (at 4-mo intervals), two ejaculates in succession were collected by artificial vagina and, having met the minimum criteria, were pooled and extended to 50×10^6 sperm/mL with clarified egg yolk-citrate-glycerol. The extended semen was packaged and cryopreserved in 0.5-ml French straws (Instruments de Médecine Vétérinaire, l'Aigle, France) according to the established optimum procedures for this extender system (Robbins et al., 1976). At the time of AI, semen was thawed by plunging the straw into 35°C water for 45 s. Throughout experiment 1, the three bulls were maintained on a weekly semen collection schedule, which consisted of harvesting two ejaculates in succession each Monday morning. This allowed for the quantitative and qualitative stabilization of epididymal sperm reserves, the monitoring of semen quality, and estimation of ejaculate characteristics of natural service to be performed later the same week.

System for Detecting Estrus, AI, and Natural Service

All cows were monitored continuously for behavioral estrus with HeatWatch (Walker et al., 1996). Beginning 44 h after the $PGF_{2\alpha}$ injection, the HeatWatch software was reviewed every 3 h to monitor the onset of estrus as defined by the time of the first standing event.

In experiment 1, cows were artificially inseminated with one 0.5-ml dose of semen (25×10^6 sperm) from one of the three bulls at onset of estrus, 12 h after onset, or received natural service at 0 h (**Nat 0 h**) from one of three bulls. Natural service was included in experiment 1 as a control to determine the effect of natural mating at onset of estrus on number of accessory sperm per embryo (ovum), fertilization rate, and embryo quality. The cryopreserved semen used was obtained within the same 4-mo period of cows receiving natural service to the same bull. For natural service, cows were restrained in a chute and one bull was allowed a single intromission and ejaculation. In experiment 2, all cows received AI with one 0.5-ml dose of semen (25×10^6 sperm) from one of the three bulls at either 0, 12, or 24 h after onset of estrus. For each of the three bulls, straws from the three frozen ejaculates were pooled and drawn at random for each insemination. Due to logistics of monitoring the computer and animal retrieval from pasture, actual times of insemination (mean ± SD) after onset of estrus were as follows: Nat 0 h, 2.3 ± 1.1 h; AI 0 h, 2.0 ± 0.9 h; AI 12 h, 12.1 ± 0.6 h; AI 24 h, 24.2 ±

0.7 h. During both experiments, cows were assigned randomly to one of the treatments.

Embryo Recovery and Evaluation

Embryos and ova were recovered by using standard nonsurgical uterine flushing techniques (Wright, 1981) 6 d after insemination for AI 12 h and AI 24 h and 7 d after insemination at AI 0 h and Nat 0 h. The initial search and evaluation of embryos and ova was performed with a stereomicroscope at magnifications of 10× to 70×. Recovered viable embryos (morulae) were evaluated at 70× magnification and, based on compactness and homogeneity of the cell mass (Lindner and Wright, 1983), were classified as either excellent, good, fair, or poor. No blastocysts were recovered. Embryos with blastomeres that contained nuclei but were too underdeveloped to be considered viable according to Lindner and Wright (1983) were classified as degenerate. An unfertilized ovum was designated when there was no indication of cleavage, or when cytoplasmic fragments were without nuclei. Throughout both experiments, two experienced people evaluated the same embryos and a consensus of quality was achieved.

Accessory Sperm Evaluation

Accessory sperm per embryo were visualized by using the procedure of DeJarnette et al. (1992). After partial digestion of the zona pellucida with 0.5% protease (Pronase; Behring Diagnostics, La Jolla, CA) in a hanging drop preparation, the embryo was compressed with a coverslip on a siliconized microscope slide, and the smear was examined with differential interference contrast optics at a magnification of 500×. This procedure (DeJarnette et al., 1992) rendered the heads of accessory sperm flat to the viewer, which facilitated an objective direct count.

Statistical Analyses

Chi-square analyses were used to test the effect of treatment on fertilization rate and embryo quality. Due to the subjective nature of judging embryo quality, embryos were grouped into three categories for χ^2 analysis, i.e., degenerate embryos, poor to fair embryos, and good to excellent embryos (Nadir et al., 1993).

DeJarnette et al. (1992) first reported the skewed distribution associated with accessory sperm data and recommended that median accessory sperm values were more important than mean values. Therefore, median accessory sperm numbers per embryo or ovum were analyzed for treatment differences by using the Wilcoxon two-sample test (SAS).

RESULTS AND DISCUSSION

The interval from the prostaglandin injection to the onset of estrus was 64.6 ± 15.5 h (mean ± SD) with a range of 36 to 96 h. The duration of estrus and number of standing events per estrus (mean ± SD) were 11.4 ± 5.1 h and 19.3 ± 16.1, respectively. The time from administration of $PGF_{2\alpha}$ to the first standing event appeared to be shorter in our experiment using nonlactating cows compared with the previously reported time of 73.1 h for lactating, single-ovulating cows (Walker et al., 1996). In addition, behavioral estrus appeared to be longer (11.4 h vs. 9.5 h) and the mean number of standing events seemed to be greater (19 vs. 10) compared with data of Walker et al. (1996). Those differences are likely due to lactational status, numbers of herdmates simultaneously in estrus, or footing surface.

Experiment 1

From 150 inseminations, 115 embryos and ova (n = 39, AI 0 h; n = 39, AI 12 h; n = 37, Nat 0 h) were recovered by using nonsurgical uterine flushing techniques 6 or 7 d after insemination. Fertilization rates were 67% (AI 0 h), 79% (AI 12 h), and 98% (Nat 0 h) and differed between AI 0 h and Nat 0 h ($P < 0.05$) and AI 12 h and Nat 0 h ($P < 0.05$, Table 1). Embryo quality was not affected by time of AI or natural service, as the percentages of excellent to good, fair to poor, and degenerate embryos were 62, 31, and 8 (AI 0 h), 61, 29, and 10 (AI 12 h), and 78, 11, and 11 (Nat 0 h) (Figure 1). Median accessory sperm values were 1 (AI 0 h), 10 (AI 12 h), and 27 (Nat 0 h) and differed between AI 12 h and Nat 0 h ($P = 0.05$), and AI 0 h and Nat 0 h ($P < 0.01$, Table 1). The reason Nat 0 h was superior in fertilization rate and median accessory sperm number may be related to 1) the ability of recently ejaculated sperm to remain fully functional longer than cryopreserved sperm from onset of estrus to ovulation (27.6 ± 5.4 h; Walker et al., 1996), 2) the high volume or components of seminal plasma and the number of sperm in the whole ejaculate, or 3) sperm selection by the cervix and mucus, increasing the population of morphologically normal, and potentially more competent, fertilizing sperm.

Experiment 2

From 154 inseminations, 117 embryos and ova (n = 39, AI 0 h; n = 39, AI 12 h; n = 39, AI 24 h) were recovered 6 or 7 d after insemination. In contrast to experiment 1, fertilization rates did not differ among treatment groups, although an apparent upward trend in fertilization rate is evident when comparing AI 0 h (66%) with AI 24 h (82%) ($P = 0.12$, Table 1). Embryo

Table 1. Effect of insemination time and natural service of nonlactating dairy cattle on accessory sperm per embryo (ovum) and fertilization rate of recovered embryos (ova).

Treatment	n[1]	Accessory sperm per embryo (ovum) Median	Mean ± SD	Range	Fertilization rate, %
Experiment 1					
AI 0 h	39	1[a]	14.9 ± 33.4	0–162	67[a]
AI 12 h	39	10[a]	36.3 ± 59.0	0–216	79[a]
Nat 0 h[2]	37	27[b]	74.4 ± 99.9	0–340	98[b]
Experiment 2					
AI 0 h	39	1[a]	9.5 ± 23.1	0–120	66
AI 12 h	39	2[a,b]	21.2 ± 46.2	0–198	74
AI 24 h	39	4[b]	33.0 ± 52.7	0–209	82

[a,b]Values within an experiment and within a column with different superscripts differ ($P < 0.05$).
[1]Number of embryos (ova) recovered.
[2]Nat 0 h = natural service at 0 h after the onset of estrus as determined by HeatWatch.

quality was affected by time of AI as the percentages of excellent to good, fair to poor, and degenerate embryos were 77, 15, and 8 (AI 0 h), 52, 38, and 10 (AI 12 h), and 47, 19, and 34 (AI 24 h) ($P < 0.05$, Figure 2).

These results strongly suggest that AI 12 h after the onset of estrus provides a compromise between a potentially lower fertilization rate (AI 0 h) and lowered embryo quality due to increased degenerate embryos (AI 24 h). From these data, pregnancy rates at one service would be expected to be optimized at AI 12 h. This agrees with the field study of Dransfield et al. (1998), in which the optimal time of AI for cows identified in estrus by HeatWatch was between 4 to 16 h after the onset of estrus, based on pregnancy rates determined by palpation.

Median accessory sperm values were 1 (AI 0 h), 2 (AI 12 h), and 4 (AI 24 h) and differed between AI 0 h and AI 24 h ($P < 0.05$, Table 1). The estimated time required for sustained sperm transport is between 6 to 12 h (Hawk, 1987; Hunter and Wilmut, 1983; Wilmut and Hunter, 1984). In the AI 0 h group, it is possible that many sperm were removed from the tract, not only through initial retrograde loss (Mitchell et al., 1985), but also through the loss of functional sperm reservoirs over the time span from insemination to ovulation. Apparently, AI 24 h allowed enough time for sustained sperm transport, and perhaps less time for loss of sperm from the functional sperm reservoirs, as evidenced by the increase in median accessory sperm number per embryo and the parallel tendency toward a higher fertilization rate.

Embryo quality in the AI 24 h group may have been impaired due to 1) inadequate time for sperm selection, or 2) fertilization of an aging ovum. The fertilizable lifespan of the bovine ovum has been estimated at up to 24 h after ovulation (Thibault, 1967), whereas the functional lifespan, during which normal fertilization

Figure 1. (Experiment 1). Embryo quality was not affected by time of AI or natural service in nonlactating dairy cattle ($P > 0.05$). Nat 0 h = natural service after onset of estrus.

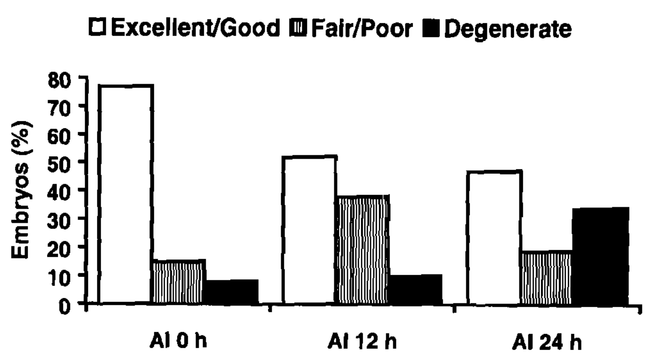

Figure 2. (Experiment 2). Effect of AI at 0, 12, or 24 h after the onset of estrus on embryo quality in nonlactating dairy cattle. The distribution of embryo quality as a result of AI 24 h is different than AI 0 h and AI 12 h ($P < 0.05$), as determined by a χ^2 test of independence using a 3 (treatment) × 3 (embryo quality) table. There is no difference in the distribution of embryo quality when comparing AI 0 h and AI 12 h ($P > 0.05$), using a 2 (treatment) × 3 (embryo quality) table.

results in a viable embryo, has been suggested to be less than 8 to 12 h after ovulation (Brackett et al., 1980; Casida, 1950; Hunter, 1988). The distinction between the functional and fertilizable lifespan of ova is important, because delayed fertilization has been shown to lead to an increase in the incidence of degenerate embryos recovered from superovulated cattle (Schiewe et al., 1987), possibly due to decreased normal ova activation competence (Ducibella, 1998). The improved embryo quality associated with AI 0 h suggests that the duration of sperm residence in the female reproductive tract may allow further selection pressure favoring competent sperm, thus optimizing embryo quality at this early insemination. The high proportion of excellent to good embryos resulting from AI 0 h would be expected to establish pregnancies at the highest rate (Lindner and Wright, 1983). This agrees with Pursley et al. (1998) regarding pregnancy loss after timed AI of lactating cows following the OvSynch protocol. According to Pursley et al. (1998), cows inseminated at the time of the second injection of GnRH (0 h), approximately 24 to 32 h before ovulation, had the lowest pregnancy loss when compared with cows inseminated 8, 16, 24, or 32 h after the second injection of GnRH.

The apparent upward trend in fertilization rate and increase in accessory sperm number from AI 0 h to AI 24 h, coupled with the least desirable embryo quality after AI 24 h, appears to contradict Nadir et al. (1993) in which embryo quality was positively associated with accessory sperm number. The biological reason these studies differ may be related to differences in the time of insemination between the studies. Nadir et al. (1993) strove to breed within 12 h after onset of estrus based on visual observations. It is possible that the lack of a positive relationship between embryo quality and accessory sperm number (as seen in the AI 24 h group of the present study) was not evident in the work of Nadir et al. (1993) because cows were inseminated less than 24 h after the onset of estrus in the latter study. A review by Saacke et al. (2000) of accessory sperm data from more than 800 embryos recovered from single-ovulating cows bred following visual observation of estrus and the a.m.–p.m. guideline indicates that 10 to 20 sperm per embryo are required before embryo quality is maximized, i.e., embryos with less than 10 accessory sperm have a higher tendency to be of low quality.

CONCLUSIONS

Natural service at 0 h increased fertilization rate and median accessory sperm number compared with AI at 0 or 12 h after onset of estrus; however, embryo quality was unaffected (experiment 1). The use of natural service provided insight into what may occur naturally and suggested that potential benefits would come from more research designed to optimize sperm preservation technology or identification of biologically important components of neat semen that would favor the functional life of sperm in vivo. This study should not be interpreted as a recommendation to return to the use of natural service because of detrimental effects upon genetic progress and animal health.

In experiment 2, AI 24 h increased median accessory sperm number compared with AI 0 h. Embryo quality was adversely affected after AI 24 h, whereas an apparent increasing trend in fertilization rate was evident from AI at 0, 12, and 24 h. Clearly, AI 12 h after onset of estrus is the recommended time for insemination, as this provides a compromise between a potentially lower fertilization rate (AI 0 h) and lowered embryo quality due to increased degenerate embryos (AI 24 h). Future efforts to improve results of AI should focus upon improving sperm access to the ovum (increasing number of accessory sperm per embryo and fertilization rate) at inseminations early in estrus when embryo quality is already favored.

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Timing of Insemination for Dairy Cows Identified in Estrus by a Radiotelemetric Estrus Detection System

M.B.G. DRANSFIELD,* R. L. NEBEL,*,[1] R. E. PEARSON,* and L. D. WARNICK†
*Department of Dairy Science, Virginia Polytechnic Institute and State University, Blacksburg 24061-0315
†Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

ABSTRACT

The optimal time of artificial insemination (AI) was determined from data for 2661 AI in 17 herds utilizing a radiotelemetric system for estrus detection that has the potential for continuous 24-h surveillance to monitor behavioral events associated with estrus. The system consisted of pressure-sensitive radio frequency transmitters affixed over the sacrum region of cows. The activation of the sensor sent a radiotelemetric signal to a microcomputer via a fixed antenna. Cow identification, date, time, and duration of each standing event were recorded in the software program provided with the system. Each farm selected a 3-h interval to AI for cows that were identified in estrus during the previous 24 h. Pregnancy status was determined from data for return to estrus and palpation of the uterus 35 to 75 d following AI. Standing events during estrus averaged (± SD) 8.5 ± 6.6 per cow, and the number of events per estrus across herds averaged from 6.2 ± 5.1 to 12.8 ± 9.9 per cow. The duration of estrus ranged from 5.1 ± 3.8 to 10.6 ± 6.8 h across herds; the mean was 7.1 ± 5.4 h. The interval from the first standing event to AI affected the probability of pregnancy; the highest conception rates for AI occurred between 4 and 12 h after the onset of standing activity. The probability of pregnancy was higher for cows >100 d in milk, exhibiting >2 standing events during estrus, and inseminated during March, April, or May.

(**Key words**: artificial insemination, telemetry, estrus, detection of estrus)

Abbreviation key: **CI** = confidence interval, **HW** = HeatWatch® (DDx Incorporated, Denver, CO).

INTRODUCTION

The adoption by dairy producers has made AI one of the most important technologies of this century; AI has been important in reducing disease transmission, allowing for genetic selection, and ultimately increasing the health, longevity, and yield of dairy cows. However, an estimated annual loss of >$300 million to the US dairy industry because of the failure to detect estrus or the misdiagnosis of estrus has reduced the positive economic impact of AI (25). Thus, the efficient and accurate detection of estrus and the timing of resulting AI remain major challenges to improving reproductive and economic efficiencies of many dairy farms (7, 8, 20, 25, 28, 29).

The timing of AI relative to the stage of estrus has been investigated for >50 yr. During the early development of the AI industry, several studies (28, 29) were designed to determine the optimal time of AI. Those studies indicate that maximum conception rates were achieved from mid estrus until a few hours after the end of expression of standing behavior, which led to the establishment of the a.m.-p.m. management guideline. This guideline for AI states that cows in estrus during the a.m. should be submitted for AI during the next p.m., and cows in estrus during the p.m. should be submitted for AI during the next a.m. (29). Two recent large field trials (8, 20) using professional AI technicians have shown that pregnancy rates using once daily AI schedules were similar to AI following the a.m.-p.m. guideline. The first trial used fresh semen and inseminated 44,707 cows either the same a.m. of observation, between 1200 and 1800 h on the day of observation, or on the following a.m. for cows identified after 1800 h (8). There was no difference in nonreturn rates at 150 to 180 d for cows that received AI the same a.m. or during the p.m. following a.m. detection. The second study (20), performed with frozen semen and 7240 first service AI, determined that nonreturn rates resulting from once daily AI did not differ from nonreturn rates for AI following the a.m.-p.m. guideline.

The optimal time of AI was predicted using mathematical models based on pedometer readings and rec-

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[1]Reprint requests.

tal palpation of 171 cows (18). The chance of pregnancy was highest between 6 and 17 h after increased pedometer activity, and the calculated optimum time of AI was 11.8 h. Unfortunately, studies designed to evaluate the optimal time of AI generally had two technical deficiencies: inadequate numbers of cows for valid statistical comparisons (18, 28, 29) and inaccurate knowledge of the onset of estrus because of low frequency and efficiency of methods used for estrus detection (8, 20).

Biological events that affect the aspect of timing of AI and fertilization are the functional viable life of gametes (sperm and ova), the transport time of viable sperm from the site of AI to fertilization, and the timing of ovulation in association with AI. Using intrarectal ultrasonography to detect ovulation and the HeatWatch® (**HW**; DDx Incorporated, Denver, CO) estrus detection system to determine the onset of standing activity associated with estrus, the interval from the first standing event of estrus to ovulation was determined to be 27.6 ± 5.4 h (30). The transport of viable spermatozoa to the oviducts requires a minimum of 6 h to obtain a population capable of fertilization, and sperm numbers progressively increase over 8 to 18 h (13, 27, 32). The functional viable life of bovine spermatozoa in the reproductive tract has been estimated at 24 to 30 h (28, 29). Although the maximum time that the ovum may retain its capacity for fertilization is 20 to 24 h, the optimum period is remarkably transitory and is estimated to be 6 to 10 h (2). Thus, with the availability of a 24-h surveillance system to monitor behavioral events associated with estrus, it seems appropriate to reexamine the timing of AI of dairy cows. The objectives of this study were to evaluate the timing of AI of dairy cows located in 17 herds that utilized the HW system to identify and record precisely the first mount of standing estrus and to characterize estrus periods for cows monitored with the HW estrus detection system.

MATERIALS AND METHODS

Herds

A variety of herd sizes (56 to 556 lactating cows) and management styles were represented by the 17 dairy farms that participated in this study (Table 1). Three of the 17 herds employed three times per day milking; 14 of the herds were on a DHI testing program and had rolling herd averages ranging from 7330 to 10,847 kg/yr. This study was conducted between July 1995 and June 1996, and no effort was made to balance data across herds and seasons.

TABLE 1. Profile of herds that participated in trial.

Herd	Cows	Housing[1]	Conception rate[2]	Daily milking frequency	DHI rolling herd average for milk
	(no.)		(%)		(kg)
1	393	C	53.0	2	9785
2	477	C	41.9	3	10,227
3	146	F	45.8	2	9324
4	106	P	44.1	2	7579
5	556	F	41.4	3	10,847
6	142	P	49.2	2	7331
7[3]	97	P	53.4	2	
8	131	F	47.4	2	8399
9	188	C	31.1	3	10,862
10	85	F	54.4	2	9356
11[3]	99	F	44.9	2	
12	82	P	55.3	2	9655
13[3]	64	F	44.9	2	
14	225	F	41.8	2	8675
15	56	F	38.2	2	7769
16	145	F	52.4	2	9039
17	228	P	43.5	2	7330

[1]Housing classification: C = total confinement, F = free stall and drylot, P = pasture and free stall.

[2]Conception rate = number of cows diagnosed pregnant divided by total number of cows inseminated.

[3]Herd did not participate in the DHI program.

System for Identifying Estrus

The HW system was utilized to detect the onset of estrus and to record standing events associated with estrus. Radio frequency data communications is the base technology employed by the HW system. The radiotelemetric device that was attached to each cow consisted of a miniaturized radiowave transmitter, powered by a lithium 3-V battery and linked to a pressure sensor enclosed in a hard plastic case 5.3- × 8.1-cm and 1.8 cm in height. Each device was secured in a water-resistant pouch and was attached to a 35- × 20-cm saddle-shaped nylon mesh patch that was glued with contact-type adhesive (DDx Incorporated) to the hair caudal to the sacral region.

Activation of the pressure sensor by weight of a mounting herdmate for a minimum of 2 s produced a radiowave transmission (0.4-km range). Transmitted data consisted of sensor identification, date (month, day, and year), time (hour and minute), and duration of sensor activation. Transmitted signals were sent to a microcomputer via a fixed radio antenna. The remote signal receiver was centrally located on each farm to minimize transmission interference. Transmitted data from the remote receiver were chronologically stored in a buffer external to the microcomputer and transferred to a microcomputer at the request of the HW software. The HW software generated both fixed management reports and individual cow files that could be viewed or printed. Individual herd files of all recorded events emitted by transmitters were electronically copied, decoded into standard ASCII files, sorted by cow into individual periods of estrus activity, and scrutinized. The HW software classified a standing estrus as occurring when a cow had three standing events in any 4-h period; fewer standing events were noted as a suspected estrus, and visual observation for secondary signs of estrus prior to the decision to AI was recommended. The herd manager or inseminator made the ultimate determination of estrus and the decision to perform AI. Inseminations were performed daily during a 3-h period chosen by each farm manager for cows identified in estrus during the previous 24 h. Because spontaneous estruses and estruses induced by $PGF_{2\alpha}$ do not differ in time from the first standing event recorded by the HW system to ovulation (30), spontaneous and hormonally induced estruses were not categorized.

Pregnancy status was determined by return to estrus or palpation of the uterus by the herd veterinarian 35 to 75 d following AI. Data for time of AI, pregnancy status, individual cow standing activity, and AI information (service sire, lactation number, and calving date) were collected bimonthly from herd personnel and HW software.

Statistical Analyses

The association between several explanatory variables and the probability of pregnancy was analyzed using the logistic procedure of SAS® (24). This procedure uses the maximum likelihood method to fit linear logistic regression models for binary response variables. The statistical significance of each explanatory variable was evaluated using likelihood ratio tests. The change in −2 log likelihood between the full model including the factor of interest and a reduced model without the factor was calculated. This value was then compared with the results of a chi-square distribution; the degrees of freedom corresponded to the change in the number of parameters estimated by the two models (16). The cows determined to be in estrus with <3 standing events that were recorded by HW were included in the logistic regression analysis. Explanatory variables included effects of herd, AI interval relative to onset of estrus, number of standing events per estrus, DIM at AI, and season. Intervals from the initial standing event of estrus to AI were divided into seven categories by 4-h increments. The number of standing events per estrus were categorized into three groups (<3, 3 to 15, and >15 standing events). Effects of season were evaluated according to climatic data for mean daily temperature from records provided by the National Oceanic and Atmospheric Administration (Blacksburg, VA). Months of the year were grouped into seasons by similar daily mean temperatures and categorized as follows: season 1 = November, December, January, and February; season 2 = March, April, and May; season 3 = June July, August; and season 4 = September and October. In order to evaluate the potential consequences of having observations from the same cow in the ordinary logistic regression model, the logistic regression results were checked using a random effects logistic regression model with cow included as a random effect (19).

Parameter estimates of the logistic regression model were used to calculate odds ratios, which are a measure of the strength of association between explanatory and response variables (16, 23). Odds ratios were interpreted as the odds of pregnancy occurring for a particular explanatory variable category relative to the baseline category for that variable when the other explanatory factors were controlled for in the model: 1, no effect on pregnancy; >1, increased probability of pregnancy; and <1, a decreased

probability of pregnancy compared with the baseline category. The 95% confidence intervals (**CI**) were calculated to show the precision of odds ratio estimates. A CI that contained the numerical value of 1.0 suggested no significant difference between the category and the baseline category for that variable. Arithmetic means were calculated for duration of estrus and number of standing events per estrus, and Tukey's Studentized range tests were used to determine the differences among means.

RESULTS AND DISCUSSION

Characteristics of the 17 dairy farms represented diverse management styles from total confinement feeding and housing systems to a system of primarily grazing (Table 1). The varied herd size (56 to 556 lactating cows) of farms participating in the trial represented much of the range in cow numbers found on Virginia dairy farms. The mean conception rate for the 2661 AI of 1616 cows was 45.3% and ranged across farms from 31.1 to 55.3%, which was similar to the 46% first AI conception rate for all Virginia dairy farms participating in DHI (n = 549) during the study. Rolling herd averages for milk yield were >10,000 kg in three herds; these herds also were milked three times daily, and two of these herds used a total confinement system of feeding and housing. No relationship was revealed between the conception rate and herd size, housing type, milking frequency, and milk yield using backwards stepwise variable selection.

Profile of Sexual Behavior

This study is the first quantitative examination of estrus characteristics across many herds using a device designed to monitor continuously the standing activity that indicates estrus. Three herds that removed the transmitters prior to the end of estrus as an attempt to prevent transmitter loss were not included in this analysis. The profile of estrus characteristics monitored by HW is presented in Table 2. The overall mean (± SD) number of standing events per estrus was 8.5 ± 6.6 (n = 2055), which was comparable with that observed (9.5 ± 6.9) for 88

TABLE 2. Profile of estrus characteristics identified by HeatWatch® electronic estrus detection system[1] for cows within herd.

Herd	Characteristics of estrus				
	Estrus period[2]	Standing event		Duration[3]	
	(no.)			(h)	
		$\overline{X}$	SD	$\overline{X}$	SD
1	362	7.7	7.2	7.3	5.8
2	351	8.2	6.4	6.9	4.9
3[4]	176	6.0	5.0	5.8	5.6
4[4]	115	5.5	4.0	5.2	4.6
5	307	7.4	6.1	6.6	4.8
6	128	8.2	5.8	8.1	5.6
7[4]	55	3.8	2.4	4.5	5.9
8	202	8.7	7.0	6.4	4.8
9	125	6.4	4.4	6.5	6.6
10	68	9.4	6.1	6.9	4.2
11	76	12.8	9.9	7.8	5.2
12	36	12.0	10.6	8.0	6.0
13	80	7.1	5.1	5.5	5.0
14	63	8.7	6.9	7.9	6.5
15	32	6.2	5.0	5.0	3.8
16	20	8.4	4.0	10.6	6.8
17	205	8.2	8.0	6.3	5.7
Total[5]	2055	8.5	6.6	7.1	5.4

[1]HeatWatch® electronic estrus detection system (DDx Inc., Denver, CO).

[2]Estrus periods with only one standing event removed from analysis.

[3]Duration of estrus defined as time interval in hours from first standing event to last standing event as record by HeatWatch® system.

[4]Transmitters were removed voluntarily after cows were identified in estrus.

[5]Herds that removed transmitters prior to end of estrus were not included in totals.

estrous cycles from lactating cows in a research herd using the HW system (30). Standing events per estrus ranged from 6.2 ± 5.0 to 12.8 ± 9.9. The conception rate was lower ($P < 0.05$) for cows (n = 260) that received AI after only one recorded standing event by HW than for cows (n = 2401) that received AI after exhibiting ≥2 standing events (36% vs. 46%). Cows that were identified in estrus and received AI after only one standing event that was recorded by HW were removed from the data set prior to calculation of means for estrus characteristics to account for cows that were possibly not in estrus.

The two herds representing the range in standing events per estrus utilized a system of free stall and drylot housing. However, the herd with the fewest standing events per estrus (herd 15) also had the fewest number of cows. Using continuous observation by video recording, Walton et al. (31) reported 8.8 standing events for cows following treatment with cloprostenol to induce estrus and 5.5 standing events for spontaneously occurring estrus in lactating Holstein cows. Factors related to environment, nutrition, herdmates, and condition of feet and legs dramatically affected the behavioral characteristics of estrus and may explain herd variation of the estrus characteristics that were monitored by the HW system (3, 4, 6, 10, 14, 17). The duration of estrus, defined as the time interval from first to last standing event, ranged from 33 min to 35.8 h, and the overall duration across herds was 7.1 ± 5.4 h. Duration of estrus was not determined for cows (n = 260) that were inseminated following only one standing event. Additionally, three herds were excluded from analysis because transmitters were routinely removed once estrus was identified to prevent the loss of the transmitter by mounting activity. As with standing activity per estrus, the duration of estrus across herds did not differ (5.0 ± 3.8 to 10.6 ± 6.8 h). Duration of estrus that was based on video recording (15) varied with the number of cows in estrus simultaneously, increasing from 7.5 to 10.1 h for 1 or 3 cows in estrus at one time, respectively. Earlier studies using the HW system reported that estrus averaged 9.5 h for lactating Holstein cows (30) and 14 h for beef heifers that had synchronized estrus (26).

TABLE 3. The circadian distribution of first and last standing events of estrus (n = 2055).[1]

Item	0001 to 0600 h	0601 to 1200 h	1201 to 1800 h	1801 to 2400 h
Onset of first standing event, %	24.5	28.4	19.8	27.3
Termination of last standing event, %	24.8	27.8	23.4	24.0

[1]Standing activity identified by HeatWatch® system (DDx Inc., Denver, CO). Estrus consisting of only one standing event and when transmitter was removed voluntarily after cows identified in estrus were removed from analysis.

TABLE 4. The distribution of estrus periods categorized by intensity and duration and identified by HeatWatch® electronic estrus detection system.[1]

Estrus category[2]	Period	Distribution	Conception rate[3]
	(no.)	(%)	
Low intensity, short duration	579	24.1	45.6
Low intensity, long duration	798	33.2	45.5
High intensity, short duration	823	34.3	47.0
High intensity, long duration	201	8.4	49.8

[1]DDx Inc., Denver, CO.

[2]Low intensity is defined as an estrus containing <1.5 standing events/h; short duration lasted <7 h from first to last standing event. High intensity is defined as an estrus containing ≥1.5 standing events/h, and long duration lasted for ≥7 h from first to last standing event.

[3]Number of cows diagnosed pregnant divided by the total number of cows inseminated.

The circadian distribution of the first and last standing events of estrus is summarized in Table 3. There were no differences in the distribution of onset and end of estrus among the 6-h periods. Video recording was used to monitor behavior continuously for 80 d; 70% of the mounting activity occurred between 1900 and 0700 h (15). This observation suggested that cows were more likely to exhibit mounting activity when they were not distracted by other activities, such as feeding, milking, and barn cleaning. The onset of standing activity is hormonally controlled by elevated estradiol concentrations in the presence of low progesterone concentrations (1); the intensity and duration of standing behavior are dramatically influenced by environmental factors (3, 4, 6, 10, 14, 17).

The distribution of estrus periods having ≥2 standing events (n = 2401) by duration and intensity is presented in Table 4. The proportions of total estrus periods were as follow: low intensity and short duration, 24.1%; high intensity and long duration, 8.4%; high intensity and short duration, 34.3%; and low intensity and long duration, 33.2%. The distribution of estrus periods by intensity and duration was similar for cows that conceived (n = 1102) and for cows that either returned to estrus or were diagnosed not

TABLE 5. Logistic binomial regression for effects of interval from first standing event of estrus to AI (n = 2661) on the conception rates of dairy cows identified in estrus by the HeatWatch® electronic estrus detection system.[1,2]

Interval from onset of estrus to AI	AI	Conception rate	Odds ratio[3]	95% Confidence Interval
(h)	(no.)	(%)		
0 to 4	327	43.1	1	...
>4–8	735	50.9	1.35	1.03–1.77
>8–12	677	51.1	1.33	1.01–1.75
>12–16	459	46.2	1.12	0.83–1.50
>16–20	317	28.1	0.51	0.36–0.71
>20–24	139	31.7	0.57	0.37–0.87
>24–26	7	14.3	0.18	0.02–1.56

[1]DDx Inc., Denver, CO.

[2]$P < 0.01$.

[3]Odds ratio is the estimated odds of a cow inseminated in a particular interval becoming pregnant relative to cows inseminated from 0 to 4 h following the first standing event, controlling for the effects of herd, season, DIM, and the number of standing events per estrus. Odds ratios: 1, no effect on pregnancy; >1, increased probability of pregnancy, and <1, a decreased probability of pregnancy compared with the baseline category.

pregnant during palpation by the herd veterinarian (n = 1299). Conception rates did not differ in intensity or duration across the four categories of estrus periods. Conception rates ranged from 45.5% for cows that had estrus periods of low intensity and long duration to 49.8% for estrus periods classified as consisting of high intensity and long duration. This distribution reinforces the importance of both the frequency of visual observation and the proficiency of individuals performing visual examination in the detection of estrus. Monitoring of behavior with the HW system increased the efficiency of estrus detection in estrus-synchronized beef heifers that had fewer standing events or shorter duration of standing activity in which estrus was missed by visual observation at specific observation periods (26).

Optimal Time of AI

Logistic regression analysis for the probability of pregnancy was performed using a model including herd, interval from onset of estrus to AI, standing events per estrus, season, and DIM at AI. The results of the logistic regression model for random effects, with cow included as a random effect, showed the correlation of observations from the same cow to be nonsignificant (P = 0.13). Therefore, the results presented are from the ordinary logistic regression model. The interval from onset of estrus to AI influenced ($P < 0.01$) the percentage of cows that were diagnosed as pregnant between 35 and 70 d post-AI. The odds of a pregnancy resulting from AI after various time intervals following the detection of estrus are presented in Table 5. The odds of pregnancy resulting from AI increased approximately 34% for cows inseminated between 4 and 12 h after the onset of estrus compared with a baseline interval of 0 to 4 h after onset. The intervals from onset of estrus to AI >16 h were related negatively to the probability of conception. The bar graph shown in Figure 1 graphically represents the cows that were diagnosed as pregnant relative to hourly intervals from the first standing event to AI. A curvilinear relationship between the interval and pregnancy is unmistakable; conception rates were highest for cows that were inseminated from 4 to 14 h following the first standing event of estrus. Variation in conception rates by hour is readily apparent, as is the overall trend of lowered conception rates >14 h from onset of estrus. Inseminations performed between 4 and 12 h following the onset of estrus achieved a conception rate of approximately 50%; conception rate was 30% for AI performed after 16 h from onset (Table 5). From previous reports (12, 18, 20, 28, 29), near optimal conception rates would be expected for cows that were submitted for AI 12 to 18 h after the detection of estrus. The frequency of visual observation prevented the accurate determination of estrus onset in previous studies (8, 12, 18, 20, 28, 29); therefore, the ability to identify the first standing event of estrus consistently and accurately with the HW system should allow for accurate timing of AI. Our results agree with those of

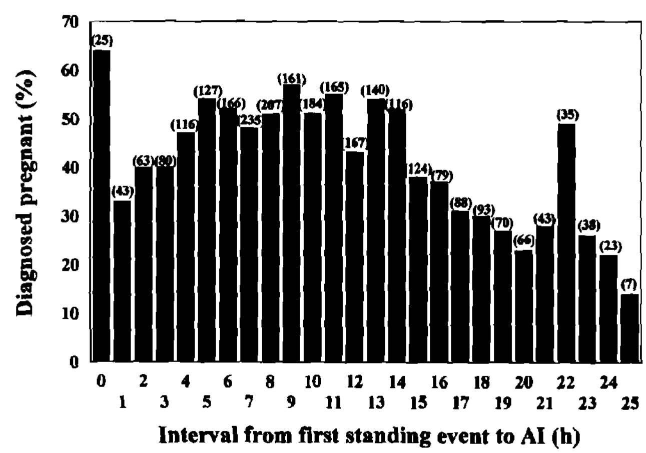

Figure 1. Percentage pregnant by hour relative to timing of AI from first standing event detected by HeatWatch® system (DDx Inc., Denver, CO) across 17 herds. Number of AI per period is within parentheses.

others (11, 21) who reported no difference in conception rates for cows and heifers that were submitted to AI shortly after the detection of estrus or 12 h later. Mathematical modeling to predict the optimal time for AI using activity pedometers and visual signs of estrus, estimated 11.8 h from onset, which coincides with the approximate midpoint of 4 to 16 h as optimum using HW (18).

The logistic binomial regressions for season, DIM at AI, and total standing events per estrus with conception rate are shown in Table 6. Season affected the pregnancy rate; the expected conception rate ($P < 0.05$) was higher for AI performed during March, April, and May. The odds ratio for pregnancy occurring within this period was 1.45, and the 95% CI was 1.12 to 1.86. The odds of pregnancy for cows inseminated during summer months were 12% lower than for AI performed during November to March. This expected drop in conception is not surprising because of the high mean temperatures for the region during the summer months and the additional heat stress experienced then. This result agrees with previous reports of higher rates of embryonic loss and lower conception rates during periods of heat stress (5). Mean daily temperature and humidity have been reported (9) to account for 80% of the variation in conception by month.

The probability of pregnancy increased as DIM at AI increased ($P < 0.01$). Inseminations occurring after 100 DIM had a greater probability ($P < 0.05$) of resulting in pregnancy; the odds of pregnancy were 46% greater than from AI prior to 75 DIM. Adverse effects of negative energy balance during early lactation have been implicated as one cause of reduced conception in early lactation (4). Reimers et al. (22) reported higher conception rates for AI in cows after 120 DIM (60%) for AI prior to 70 DIM (49%). If it is assumed that DIM and service number would follow a similar relationship, these results are similar to those of Stevenson et al. (26) that reported the pregnancy rate of beef heifers for third AI (58%) was higher than first and second AI (40%).

Interestingly, standing events per estrus also affected ($P < 0.01$) the probability of pregnancy. The baseline group of <3 standing events prior to AI, which corresponded to the activity required to activate the suspect classification in HW software, had 41% lower odds of pregnancy than cows inseminated following ≥3 standing events. Many of the cows exhibiting <3 standing events may have not been in estrus at AI, which may have contributed to a lower conception rate of cows. These findings corroborate reports of others (20, 26) and suggest that increased activity may be associated with higher conception

TABLE 6. Logistic binomial regression for effects of season, DIM, and standing events per estrus (n = 2661 AI) on the conception rates of dairy cows identified in estrus by HeatWatch® electronic estrus detection system.[1]

Category	AI	Conception rate	Odds ratio[2]	95% Confidence interval
	(no.)	(%)		
Season[3]				
September and October	477	40.7	1.0	...
November to March	1512	45.0	1.14	0.90–1.47
March to June	546	51.3	1.45	1.12–1.89
June to September	126	41.3	0.88	0.57–1.35
DIM[4]				
≤75	594	39.6	1.0	...
76–100	583	42.2	1.12	0.89–1.42
>100	1484	48.9	1.46	1.20–1.79
Standing events per estrus[4]				
≤2	601	39.1	1.0	...
3–15	1803	47.3	1.41	1.16–1.72
>15	257	46.3	1.41	1.02–1.93

[1]DDx Inc., Denver, CO.

[2]Odds ratio is the estimated odds of becoming pregnant for a cow inseminated in a particular category relative to the baseline category for that variable for the effects of the other two explanatory variables shown and for the effects of herd and interval from first standing event to AI. Odds ratios: 1, no effect on pregnancy; >1 increased probability of pregnancy; and <1, a decreased probability of pregnancy compared with the baseline category.

[3]$P < 0.05$.

[4]$P < 0.01$.

rates. Whether the estrus periods with lower activity are actually less fertile or whether many cows with <3 standing events were not in estrus at time of AI was not known.

CONCLUSIONS

Characteristics of estrus recorded by HW were highly variable and were not significantly different across herds. The onset of estrus was equally distributed during the day, and 24.1% of all estrus periods were classified as having low intensity (<1.5 standing events/h) and short duration (<7 h). These two characteristics strongly contribute to the low efficiency of estrus detection that was experienced by many dairy herds in the US. Guidelines for the timing of AI set forth by Trimberger (28) suggest approximately 12 h after observation of standing estrus as the optimal interval for pregnancy results. Results reported here would suggest that the timing of AI should be performed earlier following observation of estrus. Using the a.m.-p.m. guideline would lower the probability of resulting pregnancy, as many cows that were observed most likely had been in estrus for several hours previous to observation. Previous studies have reported that, when onset of estrus is not known, once daily AI for cows observed in standing estrus can be used as effectively as the a.m.-p.m. guideline and results in no difference in resulting conception rate (8, 20). Our results would suggest that, if onset of estrus is unknown, AI should be performed within 4 to 12 h of observation of estrus.

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Predicting Optimal Time of Insemination in Cows that Show Visual Signs of Estrus by Estimating Onset of Estrus with Pedometers

K. MAATJE,*,[1] S. H. LOEFFLER,† and B. ENGEL*,[2]
*Institute for Animal Science and Health,
PO Box 65, 8200 AB Lelystad, The Netherlands
†Animal Health Service,
PO Box 9, 7400 AA Deventer, The Netherlands

ABSTRACT

An experiment was designed to estimate the optimal interval from the beginning of estrus to artificial insemination (AI). The data were analyzed by means of a mathematical model. The analysis was based on pedometer readings and results of rectal palpation at 42 to 49 d post-AI of 171 breedings in 121 cows. The chance of conception was highest between 6 and 17 h after increased pedometer activity; the estimated optimum was at 11.8 h. In this data file, the effects of disease, inseminator, time of AI (a.m. or p.m.), and bull did not contribute to the improvement of the model. The effects of disease were not significant because of the low incidence of any specific disease. Activity measurements can be used as a tool for AI strategy to improve conception in groups of healthy cows and heifers already showing visual signs of estrus.
(**Key words**: estrus, timing of insemination, pedometers)

Abbreviation key: **CR** = conception ratio, **EDR** = estrus detection ratio, **PC** = probability of conception, **PR** = pregnancy ratio, **TI** = time from calculated onset of estrus to AI.

INTRODUCTION

Suboptimal fertility in a herd causes large economic losses to dairy farmers, second in importance only to mastitis (10, 12, 25). An important parameter of herd fertility is the pregnancy ratio (**PR**). The PR is equal to the ratio for estrus detection (**EDR**), the proportion of cows in estrus that were detected by the herdsperson, times the conception ratio (**CR**), the proportion of cows bred that were diagnosed to be pregnant at 42 to 49 d after AI (16, 31). Consequently, PR can be improved by raising either of the underlying ratios. Most herd programs for health and fertility concentrate on improving the EDR through the use of various estrus detection aids (16, 17, 20, 24, 26, 27, 32, 33). Both Senger (26) and Holz and Meinhardt (17) recently reviewed the problems and approaches to estrus detection.

The timing of AI within the estrous period can influence the CR because of the limited viability of both semen and ova (1). Macmillan and Watson (22) and Hall et al. (15) showed an improvement in CR when cows and heifers were inseminated later rather than earlier in estrus; others have shown no relationship between AI early or in mid estrus (14) and reduced CR at >16 h (29) or >24 h after the onset of estrus (12).

Two tools to measure cow activity are pedometers, which measure the actual number of steps taken by the cow in a given period, and neck transponders, which measure other types of cow activity related to estrus. Varner et al. (32) have shown that increased activity based on pedometer readings coincided closely with actual cow activity regarding the onset and cessation of standing estrus, which is the benchmark for determining time to breed. By examining the mean of 101 estrous periods, Varner et al. (32) found that when the relative activity exceeded the running mean by 100%, the onset of standing estrus followed within 2 to 4 h.

In The Netherlands, about 80% of all dairy cows are inseminated by professional inseminators who travel to farms between 0700 and 1500 h 6 d/wk. Cows reported to be in estrus before 0600 h can be inseminated the same day; those reported to be in estrus after 0600 h can only be inseminated on the following day. Using data from New York state AI organizations, without being able to verify the frequency or efficiency of estrus detection, Foote (12) found no differences between a.m. or p.m. AI and stated that a single AI of all cows to be bred in the a.m. should be near optimum. Because the onset of

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[1]Author to whom correspondence should be addressed.
[2]Agricultural Mathematics Group, Wageningen, The Netherlands.

estrus is reasonably well distributed (18) over a 24-h period (slightly higher during the nocturnal periods), it would follow that the time between onset of estrus and AI would vary considerably with such a system. The objective of this research was to examine to what degree CR is dependent on the time from onset of estrus, according to the pedometer, until AI (**TI**) for the critical period from 0 to 36 h after the onset of estrus for cows that were visually determined to be in estrus.

MATERIALS AND METHODS

Cows, Housing, and Rations

During a 2-yr period, 186 breedings from 121 cows were studied. After removal of cows with incomplete data, 171 breedings (86 in yr 1; 85 in yr 2) from 121 lactating Holstein cows and heifers remained in the analysis. The parity distribution of the cows in the study was as follows: parity 1 (n = 46), 38.0%; parity 2 (n = 38), 31.4%; parity 3 (n = 18), 14.9%; and parity 4 and higher (n = 19), 15.7%. The rolling herd average was 8650 kg of milk containing 4.6% fat and 3.5% protein during the experimental years. Ninety-six percent of the calvings was in the period from October to April. Breeding started in December and concluded in June.

Cows were housed as one group in a loose housing system with a roughened concrete floor. The floor was kept clean by a motor-driven rubber sweeper that was activated 12 times daily. Bedding consisted of a 10-cm thick straw bed (refreshed daily) on a raised concrete slab 10 cm above the walking level. The stall was well lighted with fluorescent lamps and natural light, except for a 7-h period from 2200 to 0500 h. From May to the end of June, cows were allowed on pasture between the a.m. and p.m. milkings. All cows received a basal mixed ration consisting of a corn silage, artificially dried hay, fodder beets, and a high protein concentrate; the total ration energy equivalent in milk was 27 kg (4.3% fat). Depending on production, cows were able to obtain additional (1 kg of concentrates per 2 L of milk above the basal ration equivalent) feed from automatic cow feeders. Cows had free access to the ration except during milking and pasture times.

Estrus Detection

Cows were visually observed by the herdsperson for estrus for 30 min three times daily at 0500, 1500, and 2100 h. Efficiency of visual detection of estrus on this farm during the same period was 66.7%; <1% of the results was false positive (in the period from 30 d postcalving to pregnancy) based on the larger study that included cows in the present study as well (S. Loeffler, 1996, personal communication).

Activity Measurements

Pedometers (Boumatic Heat-seeker-TX®; Dairy Equipment Co., Madison, WI) were attached to the inside of the right hind leg just above the distal expansion of the tarsal bone of each cow. The pedometers were removed from the legs once weekly (Wednesday, 0900 to 1100 h) to read data from the previous 7 d into the computer program (Boumatic Navigator II®, version 1.1; Dairy Equipment Co.). The pedometer stored data for each 2-h period for a maximum of 10 d. The pedometers were set to produce an alarm signal (flashing light) when the mean activity of the last six periods was more than double the mean activity of the last six corresponding periods for the previous 2 d (Figure 1).

If the alarm was activated, the herdsperson was able to manually determine during which period the current alarm signal had begun. This information was recorded by the herdsperson and was used to allocate the cows to a particular AI period in yr 2. Retrospectively, the exact time of the onset of estrus and the number of hours from the beginning of increased activity to the time of AI could be determined from the computer program. This period was designated as TI (Figure 1).

AI Protocol

All cows included in the trial had shown visual symptoms of estrus. During yr 1, cows were inseminated once a day during the visits of the inseminators between 0700 and 1300 h, and the exact time of AI was recorded. During yr 2, the cows were randomly assigned to one of three groups (early, average, or late onset of estrous activity) for the TI. The inseminators visited the farm three times daily (0700 to 0800 h, 1500 to 1600 h, and 2100 to 2200 h). The herdsperson was able to determine during which 2-h period the alarm signal from the pedometer had begun in order to allocate the cow to the proper group for AI. Cows in group 1 were inseminated at <10 h after the onset of increased activity, cows in group 2 were inseminated at 10 to 20 h following the onset of increased activity, and cows in group 3 were inseminated at >20 h after the first increase in activity.

Commercially available frozen semen from eight bulls with a mean nonreturn rate was chosen. A 2% difference above or below the all sire mean was al-

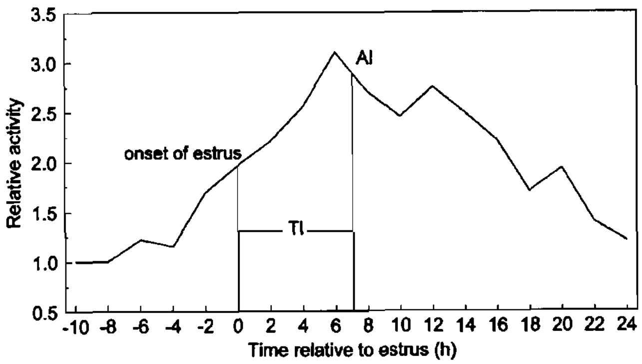

Figure 1. The relative activity (1 = basal average) of cow 377 during estrus per 2-h period. Time from onset of estrus to AI (TI) was 6.5 h. The onset of estrus was determined at the first 2-h period in which the mean pedometer reading for the current 12-h window was twice the running mean of a comparable period of the previous 2 d.

lowed. A total of eight professional inseminators was used in the 1st yr, although 78.9% of the inseminations was carried out by four inseminators. Three of these four professional inseminators completed all of the AI using only two commercially proven bulls (one lot number of each) in the 2nd yr of the study.

Cow Health

Veterinary herd health control consisted of weekly farm visits. All treatments for general health or reproduction were documented. Cows were palpated for pregnancy at 42 to 49 d after AI. The following binary variables were coded and placed into the data file together with the information related to timing of AI: retained placenta, endometritis, purulent vaginal discharge, cystic ovary, prostaglandin treatment, and feet and leg diseases. Other variables included were inseminator, bull, insemination number, parity, and length of estrous period.

Statistical Analysis

Descriptive statistics. Descriptive statistical procedures were employed to compare variables and distributions. Histograms, frequency distributions, and chi-square analyses were performed using Statistix® 4.0 (28). Results from the 1st and 2nd yr were compared before the data were pooled.

Generalized linear model for predicting optimal time of AI. Subsequent analyses were performed employing a generalized linear mixed model procedure in Genstat 5 (13) as described by Engel and Keen (9) and Engel et al. (8). Inferential procedures have been discussed elsewhere (7). The iterative reweighed residual maximum likelihood procedure used in Genstat was adapted from Keen (19).

The objective of modeling the data was to establish whether the probability of conception (**PC**) was related to TI, and, if so, for what value of TI was PC optimal?

For the generalized model, the response variable y was the binary variable for conception (0 = no conception; 1 = conception), and TI was introduced as an explanatory variable on the probit scale, together with random effects of the cow: probit (PC) = fixed effects, including TI + random effects of the cow.

Because PC, as a probability, is either 0 or 1, we could not model PC as a function of TI directly, which might have resulted in values of PC that were <0 or >1 for extreme values of TI. The probit function stretches the probabilities, avoiding the problems with boundary values 0 and 1 because the probit of PC is unbound. The probit function is well known from probit analysis, as has been described by Finney (11) for biological assays. The mixed model was chosen in the first instance to examine all breedings, because multiple breedings of the same cow cannot be seen as independent events. The random cow effects introduced into the probit model accounted for the positive correlation between breedings that corresponded to the same cow. These effects were assumed to be normally distributed with a mean of 0 and variance of σ^2.

The variables [infirmity (n = 2), parity (n = 4), inseminator (n = 8), sire (n = 8), and time of AI (n = 2; before or after 1200 h and also as a linear and quadratic covariate)] were tested by means of the Wald test adapted from Cox and Hinkley (4) and implemented in Genstat 5, as was described by Buist and Engel (2), to see whether those variables added significantly to the model. Nonsignificant fixed effects were then removed before the final model was determined.

On the probit scale, linear and quadratic terms for TI were introduced (for details see the appendix): probit (PC) $= \alpha + \beta \times \mathrm{TI} + \gamma \times (\mathrm{TI})^2$ + random effects of the cow.

An optimal value for TI (OTI) was achieved when $\gamma \neq 0$. A significance test was performed to see whether γ differed from 0. The OTI = $-\beta/(2\gamma)$; the corresponding probability was the probability of OTI = $\Phi\ [\alpha - \beta^2/(4\gamma)]$.

A 0.95 confidence interval for the optimal value of TI was constructed using the method of Fieller as described by Finney (11). A 0.95 confidence interval was also constructed for the optimal value for PC (Table 1).

TABLE 1. Estimated values for the probability of conception (PC), corresponding standard errors, and lower and upper limits of a 0.95 confidence interval for PC for various values of time from calculated onset of estrus to AI (TI).

TI (h)	PC	SE	Lower limit for PC	Upper limit for PC
5.0	0.81	0.074	0.34	0.92
6.0	0.83	0.061	0.49	0.93
7.0	0.85	0.052	0.60	0.93
8.0	0.86	0.046	0.68	0.94
9.0	0.87	0.043	0.74	0.94
10.0	0.88	0.041	0.77	0.94
11.0	0.88	0.041	0.79	0.95
12.0	0.88	0.041	0.78	0.95
13.0	0.88	0.042	0.77	0.94
14.0	0.87	0.043	0.74	0.94
15.0	0.86	0.045	0.70	0.93
16.0	0.85	0.047	0.65	0.92
17.0	0.83	0.049	0.58	0.91

Simplified linear model without a variable for the random effects of cow. For the relatively small number of AI per cow, the estimation procedure might be biased (7). Furthermore, repeated AI on the same cow involved a form of censoring because, after a successful AI, no repeat AI follow. Therefore, the analysis was repeated on a reduced data file. The reduced data file involved, for each cow, only the first available AI. The model reduced to a probit analysis model as was described by Cox and Snell (5). Results were compared with those obtained from the full data file under the generalized linear mixed model procedure (19).

RESULTS

Figure 2 displays the distribution of the cows in 8-h intervals of TI for the 2 yr. A thrice daily AI scheme during the 2nd yr was associated with more AI in the early intervals (0 to 8 h) and fewer AI in the later intervals, although this distribution was not different for the 2 yr when examined with a scale of either 4- or 8-h intervals [chi-square statistic = 8.39 ($P = 0.30$) or 3.56 ($P = 0.31$), respectively]. The mean CR also was not different between yr 1 and 2 (0.697 vs. 0.635) when compared using Student's t test ($t = 0.86$; $P = 0.39$). The mean CR (for either first AI only or all AI) was lowest for cows in parities 2 and 3 in both years. Relatively fewer parity 4 and older cows and more 2nd parity cows were used in the 2nd yr of the trial. When parity was examined per 8-h TI group, distribution was not different from that expected ($P > 0.10$, by chi-square statistic) based on overall parity distribution. The crude percentages for pregnancy for these same TI groups (first AI only) are shown in Figure 3, which shows that the pregnancy rate with TI >24 was dramatically reduced.

The frequency of abnormal veterinary findings (cysts, 3 of 121; endometritis, 8 of 121; feet and leg disorders, 23 of 121; retained placenta, 4 of 121; prostaglandin treatment, 9 of 121; and abnormal vaginal discharge, 10 of 121) was considered to be low for each variable alone, and, in some cases, more than one disease occurred in the same cow. These variables were all pooled into one binary variable infirmity, to be used in the model. This variable was equally distributed among the AI intervals (Table 2). The infirmity factor was added to the generalized mixed model with the factors of inseminator, sire, and AI prior to or after 1200 h. None contributed to the model ($P = 0.60$, 0.91, and 0.21, respectively), and all factors were subsequently omitted from the analysis.

Data were analyzed first for the full data file and subsequently for the reduced data file (first AI only). In both analyses, the γ coefficient was significantly different from 0, showing the existence of an optimal TI. Other results were also quite similar for both data files. For instance, for the reduced data file, the optimal TI was estimated at 11.8 (±1.66). The 0.95 confidence interval was 4.2 to 14.3, which was actually narrower than that for the full data file. However, closer inspection of the data showed five influential observations with a high value of TI and six influential observations with a low value of TI. From these observations, three were not included in the reduced data file. When these observations were excluded from the full analysis as well, the result was an optimal TI of 12.1 (1.5), and the 0.95 confidence

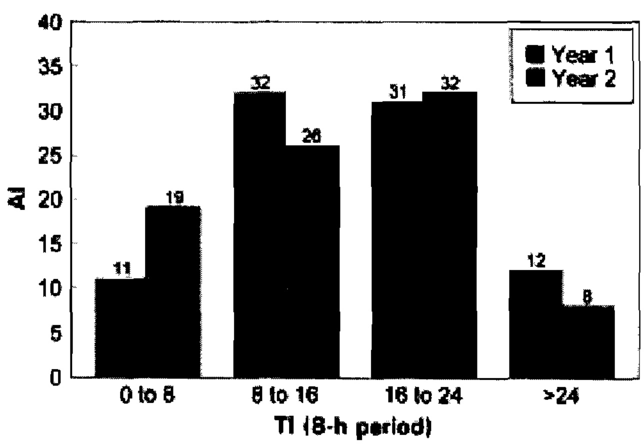

Figure 2. Distribution of time from onset of estrus to AI (TI) for the 1st yr (n = 86) and 2nd yr (n = 85) of the study. A t test for the difference between years was nonsignificant (chi-square, $P > 0.10$).

TABLE 2. Conception per period for cows with one or more abnormal or normal veterinary findings. Disease did not contribute significantly to the final model.

Infirmity status	(no.)	TI[1] Interval 0 to 8 h	8 to 16 h	16 to 24 h	>24 h
		(no. conceived/total no.)			
Normal	76	11/14	21/26	24/30	1/6
Abnormal	45	5/6	11/12	11/16	2/11
Total	121	16/20	32/38	35/46	3/17

[1]Time from calculated onset of estrus to AI.

interval was 6.0 to 14.5, which was actually somewhat narrower than that found in the reduced data file. In the analysis of the full data file, when effects of cow were not included, no significant overdispersion was found, although the P value (derived from the deviance, employing a chi-square approximation) was low ($P = 0.09$). Results from this analysis were again similar to the results mentioned previously.

Because the mixed model did not reduce the width of the estimation of the optimal TI, the straightforward probit analysis of first AI was used to model the PC for the various intervals of TI. Table 2 and Figure 4 show the PC around the optimum. As is shown, AI between 6 and 17 h after the beginning of estrus produced an estimated PC >0.83.

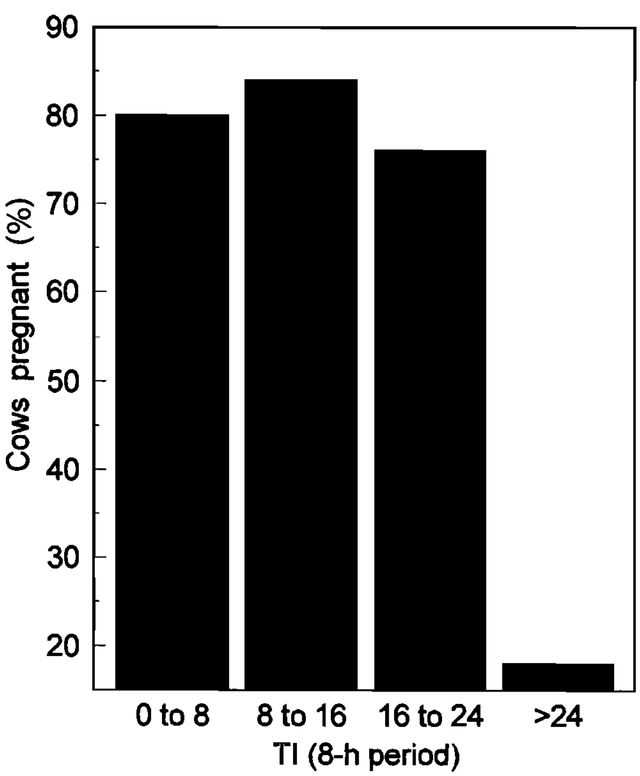

Figure 3. Crude percentage of all 121 cows that became pregnant following first AI grouped per 8-h TI (time from calculated onset of estrus to AI) value.

DISCUSSION

Proper control for the time of onset of estrus is a difficult endeavor in studies because the observations of the herdsperson are generally used to determine early or late estrus. Because peaks in estrus activity often occur at night (18), documentation of the actual onset of estrus may be difficult without 24-h observation. Therefore, studies based on normal farm practices for estrus detection might contain a selection bias (early vs. late) in the group allocation. This bias probably occurred in the study of Stevenson et al. (29) because conception rate had already decreased 16 h after the first detected estrus. Because cows were observed for estrus once daily in that study, it would follow that the onset of estrus occurred 4 to 8 h earlier than was documented. Another possible confounding aspect in some other studies (15, 22) was that fresh semen was often used, and viability differences between fresh and frozen semen may exist. However, the results of this study do not support the existence of confounding because results agree well with results of earlier studies (30) using fresh semen that found that AI at >24 h following the onset of estrus yielded poorer pregnancy results. The raw means for conception of 0.63 to 0.69 for the 1st yr can be regarded as an indication of the PC for a conventional AI strategy for which the intervals from onset of estrus to AI are more or less normally distributed (Figure 2). This distribution means a potential improvement in PR of 8 to 10% with an EDR of about 50 to 60%. When the estrus detection rate is higher, the improvement will be greater.

Because all cows that were inseminated had been observed to be in estrus, the difference in PR at 42 d post-AI for the different groups was independent of estrus detection and was more directly related to

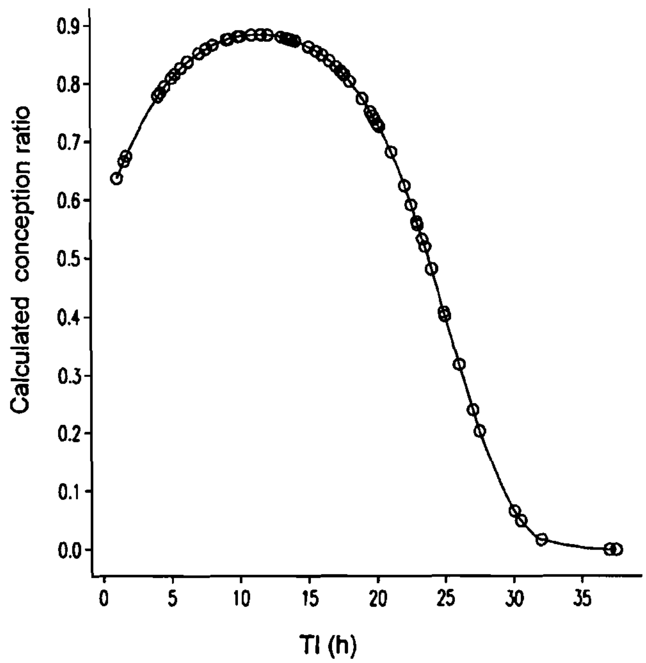

Figure 4. Probability of conception calculated from the simplified model for the reduced data file. The x-axis represents the actual values of TI (time of calculated onset of estrus to AI). The circles on the line represent the number of observations of TI at the different time intervals.

conception rate. The assumption is, of course, that any early embryonic death that is not attributable to TI was equally distributed among all TI values. Although there was an attempt to achieve a more even distribution of TI in the 2nd yr, the difference compared with yr 1 values was only apparent for cows that were inseminated very early, which explains the rather wide confidence intervals for the PC in the model for very early or very late AI.

The use of a probit model with random effects for cow provided an estimate that was similar to that of a straightforward probit model utilizing only first AI. Because of the binary nature of conception and because a series such as {0,1} but not {1,0} can exist, any assumptions about normally distributed effects of cow could be disputed. For this reason, the simpler model was chosen. In both the reduced and full data file, the γ coefficient was significantly different from 0, which indicated that an optimal TI exited. The width of the TI was sufficient for practical use in the field.

We have shown that pedometers have the potential to improve the conception rate; they indicate the onset of a ≥100% increase in activity with respect to the corresponding periods of the last 2 d, which indicates estrus, thus permitting AI of cows within 6 to 17 h. The values for standard errors could possibly be made more precise by utilizing a larger data file that would reduce the size of the 0.95 confidence intervals. By using the probit model, we were able to estimate better the optimal TI. In our data file, use of second and third AI did not improve the standard error of optimal TI. However, our model was useful in showing the slight disadvantage of early AI (<5 h after onset of estrus), and, although the number of AI was few, these results agreed with previously mentioned studies (15, 22). Furthermore, it is clear from both the crude pregnancy percentages (Figure 3) and the modeled chance of pregnancy (Figure 4) that AI at >24 h after the onset of estrus has a much poorer chance of resulting in pregnancy than AI performed between 6 to 17 h after detection of estrus. Notably, very few cows in our data had reproductive disorders at the time of breeding. Because of the weekly veterinary inspections, cows were usually diagnosed when the disorder was at an early stage, and the cows were not bred until cycling and uterine findings were deemed normal. Because of the pressures of maintaining a mean calving interval of 12.5 mo for research purposes, cows that did not return to normal gynecological status before June were not rebred. On a commercial dairy, the number of cows with disorders that are bred would undoubtedly be higher. The influence of various diseases on CR has been described by Eicker et al. (6) for cows in the state of New York.

The results of this research do not give any indication as to what degree farmers can rely on pedometers because only cows that were also observed in estrus were included in this study. Furthermore, it is well documented that pedometers can give false-positive results for detection of estrus; the number of false-positive results depends on housing, number of cows in estrus, and the threshold value or algorithm used to determine estrus (26, 32). This study utilized pedometers that recorded activity for 2-h periods because transponders that record activity per milking (12- or 8-h intervals) gave a less specific estimation for the onset of estrus. In this case, the model would have to measure the time from the first 8 or 12 h of increase in estrus activity until AI. Analyses based on these types of systems for activity measurement are currently being conducted. Nebel and McGillard (23) studied a similar interval from 6 to 18 h based on first mounts that were detected with a pressure-sensitive device. In that study, the first mount occurred about 26 h before ovulation. Our optimal value of about 12 h coincided with the midpoint of the range of Nebel and

McGillard (23). The first mount should logically occur a number of hours before the activity reaches the threshold value of twice the running average. Varner et al. (32) provided further information on increased activity and standing estrus. Chenault et al. (3) showed that peak values for bovine LH occur slightly (mean, 2.8 h) before the beginning of estrus. Because LH concentrations reliably occurred in the cow at 24 h prior to ovulation (3), our optimum of about 12 h after the onset of estrus would allow time for semen to move to oviducts and mature before ovulation. Semen that is artificially inseminated at <5 h after the onset of estrus would be more likely to lose its motility (mean viability, 24 h) by the time ovulation, transport, and ovum ripening had occurred. The AI taking place at >24 h after the onset of estrus would be more likely to facilitate semen arrival in the oviduct at a time when the ovum had already begun to deteriorate based on mean ovum viability (1).

In conclusion, the aim of most published applications of pedometers until now has been to improve rates of estrus detection. A review by Lehrer et al. (21) stated that 70 to 80% of cows in estrus are detected by pedometer measurements. From this study, the pedometer appeared to be a promising tool to determine the optimal time for AI during the estrous period. The combination of both qualities may contribute to optimal herd fertility.

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APPENDIX

As shown in the work of Engel et al. (8), this particular generalized linear mixed model is equivalent to a threshold model for a normally distributed underlying variable, z:

$$z = \alpha + \beta \times \mathrm{TI} + \gamma \times (\mathrm{TI})^2 + \text{effects of cow} + \text{residual effects.}$$

The residual effects are assumed to be normally distributed as well with a mean of 0 and a variance that without loss of generality, may be assumed to be equal to 1. The threshold value may be taken to be 0. Hence, a positive value of z corresponds with y = 1 (conception), and a negative value corresponds with y = 0. The correlation with the underlying scale between observations on the same cow is $\rho = \sigma^2/(\sigma^2 + 1)$. The larger this correlation, the greater is the dependence of the PC on the individual cows.

Because TI is estimated from a ratio, $-\beta/(2\gamma)$, the Fieller interval is more appropriate than the conventional Δ interval, which is based on a quadratic approximation of the log likelihood around the parameter estimates. For the probability of optimal TI, a Δ interval was first constructed for $\alpha - \beta^2/(4\gamma)$. This interval was then transformed using the standard normal distribution function. In this case, a plot for the reduced data file of the log likelihood against $\alpha - \beta^2/(4\gamma)$ shows a fair quadratic relationship that is close to the neighborhood of the estimate for $\alpha - \beta^2/(4\gamma)$. The final interval is virtually the same as the likelihood ratio interval, employing the chi-square distribution with one degree of freedom for the likelihood ratio test.

OUR INDUSTRY TODAY

Timing of Artificial Insemination of Dairy Cows: Fixed Time Once Daily Versus Morning and Afternoon

R. L. NEBEL, W. L. WALKER, and M. L. MCGILLIARD
Department of Dairy Science
Virginia Polytechnic Institute and State University
Blacksburg 24061-0315

C. H. ALLEN and G. S. HECKMAN
Atlantic Breeders Cooperative
1575 Apollo Drive
Lancaster, PA 17601

ABSTRACT

Nonreturn rates to professional technician service of 7240 first AI Holstein cows were calculated to evaluate differences between once daily and a.m.-p.m. AI. To determine whether management practices affected nonreturn rates, participating herd owners were surveyed for methods used for detection of estrus. Nonreturn rates for once daily and a.m.-p.m. AI were 64.6 and 65.6% for 60-d, 60.1 and 60.6% for 75-d, and 58.4 and 57.8% for 90-d nonreturn periods. Signs of estrus for AI and interval from detection of estrus to AI were related to nonreturn rates. Nonreturn rate was highest, 63.4%, when cows were in standing estrus. Nonreturn rates were lowest, 36%, when cows were bred after treatment with $PGF_{2\alpha}$ without being detected in estrus or bred strictly on veterinary advice based on palpation. Nonreturn rates were similar for different times of the day when once daily AI was practiced. However, AI in the midmorning may have some advantages. The highest nonreturn rate for a 3-h period was 68.2% for 0800 and 1100 h; the lowest was 54.7% for 1300 to 1600 h. Movement before observation for estrus and an observation period >15 min improved nonreturn rates for once daily AI. Once daily AI can be used effectively with no difference from the traditional a.m.-p.m. system; results are best when AI is based on standing estrus and performed between 0800 and 1100 h.
(**Key words**: artificial insemination, nonreturn rates, reproduction, management)

INTRODUCTION

Timing of AI relative to the stage of estrus has been under investigation for approximately 50 yr (19). Numerous studies have confirmed the conclusions of Trimberger (17) and Trimberger and Davis (19) that conception rates are maximal when cows are submitted for AI from midestrus to the end of standing estrus (7, 9, 10, 18). This early work led to the a.m.-p.m. management guideline that permits the AI of most cows near their optimal time for fertilization. This guideline for AI states that cows in estrus during the a.m. should be submitted for AI that p.m. and that cows in estrus during the p.m. should be submitted for AI the next a.m.

Fertility did not differ between single and multiple AI during estrus (6, 19, 21). Using once daily AI schedules, others (3, 15, 16) reported higher conception rates when AI was performed before 1200 h. Paternity of calves was determined to assess timing of AI (1). Semen from three sires, of three breeds, was rotated at first observation of estrus and 12 and 24 h later (1). Three AI during estrus did not result in an exceptionally high conception rate, 70.1%, and time of AI with respect to ovulation was not important to conception. The AI of cows and heifers immediately following detection of estrus (a.m. or p.m.) or 12 h later did not adversely affect conception rates (6, 16, 20). However, a wide variety of conditions, such as single AI of beef cattle (13), dairy heifers synchronized with $PGF_{2\alpha}$ (4), and the use of unfrozen semen in dairy cattle (3, 9, 10), resulted in near maximal conception rates.

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High conception rates are dependent on correctly identified estrus in cows and heifers. Liberal interpretation of indicators of estrus reduces conception rates (12, 15). Major factors affecting intensity or expression of estrus include group size, number of females in estrus simultaneously, location (dirt vs. concrete flooring), housing facilities, environmental temperatures, proper training of observers, and frequency of observation periods (2, 5, 8, 11). The main objective of this study was to establish nonreturn rates in a large sample of dairy cows submitted for AI once daily and following the a.m.-p.m. guideline with frozen and thawed semen. Additionally, because individual dairy producers using AI must interpret signs or symptoms of estrus before submitting cows for AI, a survey of methods and signs used in detection of estrus was conducted for herds participating in this field trial.

MATERIALS AND METHODS

This study involved 7240 first AI of Holstein cows from Pennsylvania dairy herds (n = 166) using Atlantic Breeders Cooperative professional technician service exclusively during 6 mo. Technicians within a service unit (<3 counties) were equally assigned to a particular AI program (once daily or a.m.-p.m.) for an initial 3-mo period and switched to the other program for the remaining 3 mo. This crossover design was utilized to minimize effects of herd and season on conception rates. Once daily AI was defined as a fixed 3-h period for AI. In the a.m.-p.m. system, cows first observed in estrus during the a.m. were submitted for AI that p.m., and cows first observed in estrus during the p.m. were submitted for AI the following a.m. A survey of management procedures used by each dairy producer for detection of estrus was conducted at initiation of the field trial (Figure 1). This survey contained questions concerning frequency and duration of observation periods for detection of estrus, interval between and location of observations, and whether devices and aids were used to assist in detection of estrus. Herd size and the 3-h period chosen for once daily AI were also recorded.

The time of day that cows were initially observed in estrus and the hour of AI were recorded for each AI. A maximum of three reasons for submitting a cow for AI could be recorded. Cows were submitted for AI based on the following indicators of estrus: 1) standing estrus, 2) mounting activity, 3) clear mucus discharge from reproductive tract, 4) fully red or missing Kamar® (Steamboat Springs, CO), 5) partially red Kamar®, 6) $PGF_{2\alpha}$ administration (72 to 80 h prior) without observed standing estrus, 7) nervousness and excessive vocalization, 8) redness and edema of external genitalia or rubbed tailhead, 9) veterinary recommendation based on ovarian palpation per rectum, and 10) other subtle symptoms or aids used for detection of estrus. Fertility was estimated from 60- to 90-d percentage of nonreturn to first AI. An indicator variable was constructed for each nonreturn interval. When a cow did not return for AI within the 60-, 75-, or 90-d interval, the variable was assigned a value of 1. A value of 0 was assigned when the cow was submitted for AI within the particular AI interval.

Independence of AI program and nonreturn interval was tested using nonreturn percentages generated from the indicator variable and a chi-square statistic. The 75-d nonreturn interval was analyzed by ANOVA (14) in a model containing herd, sire, AI program, indicators of estrus, intervals to AI, and interactions with AI program. Effects were tested by their interactions with herd. Significant effects were further compared with Tukey's pairwise contrasts. Probabilities of nonreturn were estimated simultaneously for indicators of estrus and intervals from estrus to AI using a stepwise logistic regression. Herd differences in 75-d nonreturn between AI programs were analyzed by general linear models relative to management procedures acquired by survey (14).

RESULTS AND DISCUSSION

Nonreturn rates for once daily and a.m.-p.m. AI programs are summarized in Table 1. Of most importance is the lack of difference ($P > .05$) in nonreturn rates for once daily and a.m.-p.m. AI programs at all intervals of return to estrus measured (60 to 90 d). This finding agrees with results of Gwazdauskas et al. (5), who, using one university experimental dairy herd, reported that conception rates did not differ for cows and heifers submitted to AI shortly after detection of estrus or 12 h later.

Results were also similar for dairy heifers synchronized with $PGF_{2\alpha}$ and submitted for AI following the a.m.-p.m. guideline or a.m. only (4, 6). Foote (3) reported that, with unfrozen semen, the best time for AI of dairy cattle is midmorning. Figure 2 shows 75-d nonreturn rates for once daily AI by 3-h period of AI ($P > .05$). Timing of once daily AI did not effect nonreturn rates when management practices associated with detection of estrus were con-

1. Does this farm have a routine estrus detection program?
   Yes   No
2. If a program exists, do observations occur at a prescribed time?
   Yes   No
3. If there is no program, under what conditions does estrus detection occur?
   Use of aids or devices   Casual observation
   Observed in holding area   Other
4. How many times per day are cows observed for estrus?
   Once   Twice   More than twice
5. What is the time interval between observation periods?
   12 h   <12 h   >12 h
6. How long are estrus observation periods?
   <15 min   15 min   >15 min
7. Are cows moved just prior to estrus observation period?
   Yes   No
8. Where are cows located when observed for estrus?
   Free stalls   Dirt lots   Holding pens
   Pastures   Milking parlor   Feed lots
   Other
9. Has the person who is observing cows for estrus been trained to observe secondary signs of estrus?
   Yes   No
10. Which of the following estrus detection aids or devices are used?
    ______ Calendar to record and anticipate day of estrus
    ______ Kamar® (Steamboat Springs, CO) heat mount device
    ______ Chalk, crayon, or paint smeared on tail head
    ______ Teaser animal, such as hormonally treated female, cystic cow, or surgically altered bull
    ______ Milk progesterone test to confirm estrus
    ______ Other
11. Circle the 3-h time period chosen for once daily AI.
    a.m. 0600 to 0900   0700 to 1000   0800 to 1100
    0900 to 1200   1000 to 1300   1100 to 1400
    1200 to 1500

    p.m. 1300 to 1600   1400 to 1700   1500 to 1800
12. What is the number of cows in the milking herd?

Figure 1. Questionnaire of management practices associated with a program for estrus detection.

TABLE 1. Nonreturn rates for once daily and a.m.-p.m. AI.

AI Program	Cows	Nonreturn interval 60 d	75 d	90 d
	(no.)		(%)	
Once daily	3659	64.6	60.1	58.4
a.m.-p.m.	3581	65.6	60.6	57.8

sidered and analyzed across all 3-h periods. However, nonreturn rates tended to be higher before 1200 h; actual nonreturn rates were highest for cows bred between 0800 and 1100 h or midmorning.

When the herd and the service sire were held constant, indicators of estrus and interval from detection of estrus to AI affected 75-d nonreturn rates (Table 2). The AI program had no effect on 75-d nonreturn rates. Although several of the interactions with herd (error terms) were significant, indicating variation among herds, main effects tested by them were still significant.

Cows submitted for AI based on standing estrus or secondary indicators of estrus had similar nonreturn rates (63.4 vs. 59.5%). Stevenson et al. (15) observed that cows detected by standing estrus or mounting activity had similar conception rates. Hurnik et al. (8) reported that 79% of all mounting cows and 90% of cows exhibiting standing estrus were in true estrus. Cows presented for AI because of a fully triggered Kamar® device had lower nonreturn rates than did cows detected standing or by secondary indicators. However, a fully triggered Kamar® device was a better indicator of estrus than veterinary recommendation based on presence of ovarian structures or the AI of cows given $PGF_{2\alpha}$ without observation of standing estrus ($P < .01$). This finding was not surprising, because Reimers et al. (12)

TABLE 2. Anlaysis of variance for 75-d nonreturn rates.

Source	df	MS
Herd	165	.84**
Service sire	243	.39**
AI Program[1]	1	.07
Indicator[2]	5	9.11**
Interval[3]	5	1.15**
AI Program × indicator	5	.15
AI Program × interval	5	.27
Herd × AI program	164	.33**
Herd × indicator	437	.19
Herd × interval	618	.23*
Herd × AI program × indicator	229	.25*
Herd × AI program × interval	334	.26*
Residual	4977	.20

[1]Once daily and a.m.-p.m. AI.

[2]Interval from detection of estrus to AI.

[3]Indicators of estrus.

*$P < .05$.

**$P < .001$.

TABLE 3. Influence of indicator of estrus used for submission to AI on 75-d nonreturn rate.

Indicator of estrus	AI[1]	75-d Nonreturn rate
	(no.)	(%)
Standing estrus	4622	63.4[a]
Secondary indicators[2]	1057	59.5[a]
Positive Kamar® device	575	50.8[b]
$PGF_{2\alpha}$ without observed standing estrus	126	35.8[c]
Veterinary recommendation[3]	73	35.6[c]

[a,b,c]Means with different superscripts differ ($P < .01$).

[1]Total of 746 AI with no estrus sign recorded.

[2]Mounting activity, clear mucus discharge, nervousness and excessive vocalization, redness and edema of genitalia or tail head, and partially red Kamar® (Steamboat Springs, CO).

[3]Based on ovarian palpation per rectum.

TABLE 4. Influence of interval from detection of estrus to AI on 75-d nonreturn rate.

Interval from detection of estrus to AI	AI[1]	75-d Nonreturn rate
(h)	(no.)	(%)
0 to 6	1126	59.9[a]
6 to 12	2352	60.7[a]
12 to 18	2455	55.5[b]
18 to 24	962	53.4[bc]
24 to 30	99	49.6[c]

[a,b,c]Means with different superscripts differ ($P < .01$).

[1]Total of 205 AI with no detection or AI time recorded.

observed that 10.6% of the cows with fully triggered Kamar® devices presented for AI were not in estrus, as determined by high progesterone concentrations in milk, and a 7.1% lower conception rate resulted from the AI of these cows.

Cows that were submitted for AI at 80 h following $PGF_{2\alpha}$ administration had a nonreturn rate of 35.8%. Similar results were reported by Gwazdauskas et al. (6) for cows that were submitted for AI after $PGF_{2\alpha}$ administration without observed estrus. Gwazdauskas et al. (6) reported that cows treated with $PGF_{2\alpha}$ and detected in estrus achieved a conception rate of 49.5 versus 20.7% for cows that were not observed in estrus. Cows submitted for AI as recommended by veterinary palpation of ovarian structures resulted in a 35.6% nonreturn rate (Table 3). Although nonreturn rates ($P < .01$) were higher for cows observed in standing estrus or displaying secondary signs than for cows identified as having a positive Kamar® device or after veterinary recommendation or 80 h after $PGF_{2\alpha}$ administration without estrus, approximately one of three incidences of cows submitted for AI resulted in nonreturn to AI in the latter groups. Stevenson et al. (15) suggested that liberal interpretations of indicators of estrus would increase pregnancy rates even with lower conception rates because of the low submission rates or efficiency of detection of estrus in most US dairy herds.

Nonreturn rates based on when cows were first observed in estrus ranged from 49.6 to 60.7% (Table 4). Intervals extending beyond 12 h from detection of estrus to AI reduced nonreturn rates ($P < .01$). Similar results have been reported to others (6, 9), who suggested that reduced fertility with AI 12 to 24 h after the end of estrus is probably related to lower viability of the ovum when sperm reaches the

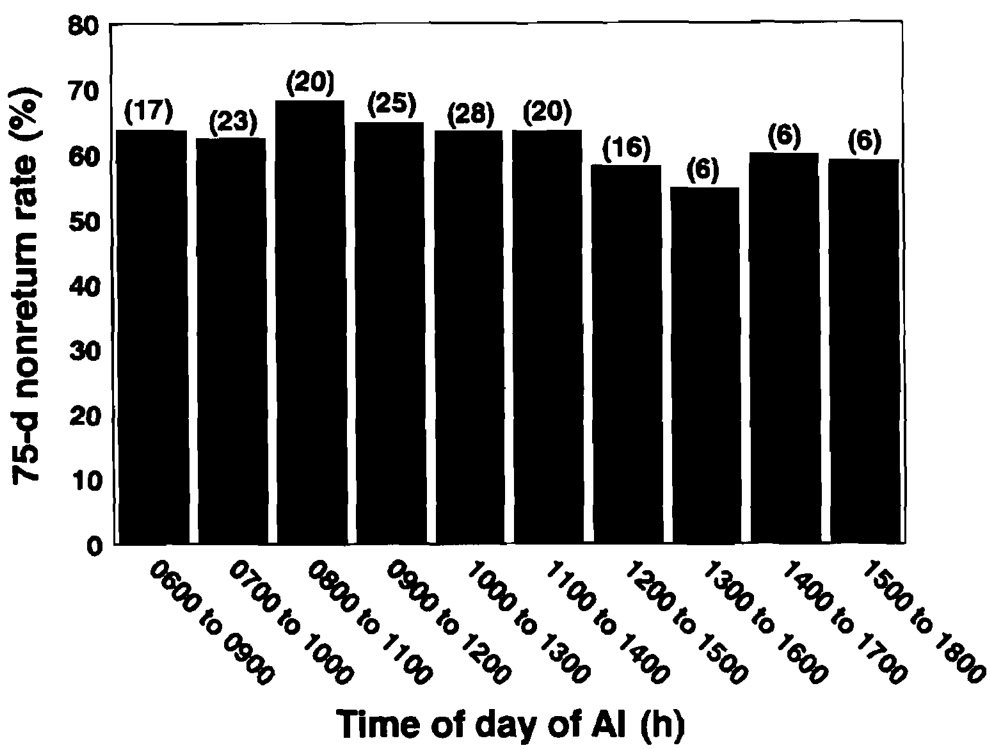

Figure 2. Nonreturn rate (75 d) for once daily AI by 3-h period of AI. Number of herds per period are within parentheses.

oviducts, a result of the time required for sustained phase of sperm transport to site of fertilization.

A stepwise logistic regression on 75-d nonreturn rates from both AI programs for indicators of estrus and intervals from detection of estrus to AI was used to determine probability of nonreturn to AI; other variables were held constant in the model. Indicators of estrus that were related ($P < .01$) to probability of conception or nonreturn were standing estrus (66.7%), AI at 80 h following $PGF_{2\alpha}$ administration without detection of estrus (41.9%), and veterinary prediction of ovulation based on ovarian palpation per rectum (40.1%). Additionally, two intervals from detection of estrus to AI were related negatively to probability of conception, 12 to 18 h (57.7%) and 18 to 24 h (53.8%). From previous reports (6, 7, 9, 10, 17, 18, 19), near optimal nonreturn rates would be expected for cows detected in standing estrus and submitted for AI 12 to 18 h after detection of estrus.

Two management practices were identified as affecting nonreturn rates obtained with once daily AI ($P < .05$). Movement of cows immediately before observing for estrus and an observation period >15 min improved nonreturn rates with once daily AI. A majority of dairy producers (71.4%) moved cows immediately prior to observation for signs of estrus; however, only 18.5% indicated that their observation period lasted >15 min.

CONCLUSIONS

When observations for estrus are frequent (every 2 to 4 h), cows should not be submitted for AI the first few hours after detection (7, 17). Under routine field conditions of less frequent observation for estrus, as reported by this study, in which only 36.9% of the dairy producers used observation periods more than twice daily, cows can be submitted for AI shortly after detection of estrus with nearly optimal results with respect to timing. Observation periods are usually associated with milking; thus, AI in midmorning, as suggested by Foote (3), when most cows have been in estrus between 12 to 18 h, probably yields the highest probability of conception. Movement of cows immediately prior to observation of estrus and an observation period >15 min are two management practices that improved nonreturn rates with once daily AI. Thus, once daily AI is an efficient system if performed during midmorning, especially when cows can be moved prior to observation periods >15 min.

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Effect of Time of Artificial Insemination on Pregnancy Rates, Calving Rates, Pregnancy Loss, and Gender Ratio After Synchronization of Ovulation in Lactating Dairy Cows

J. RICHARD PURSLEY,*,[1] ROY W. SILCOX,† and MILO C. WILTBANK*,[2]
*Department of Dairy Science, University of Wisconsin, Madison 53706
†Department of Animal Science, Brigham Young University, Provo, UT 84602

ABSTRACT

In order to assess the optimal time of artificial insemination (AI) in relation to ovulation, lactating dairy cows (n = 732) from herds with rolling herd averages of 9980 to 11,800 kg from three milkings per day were randomly assigned to five groups by stage of lactation and parity. Ovulation was synchronized by administration of GnRH followed 7 d later with $PGF_{2\alpha}$ followed 2 d later with a second treatment with GnRH. Cows were inseminated at 0, 8, 16, 24, or 32 h after the second injection of GnRH (ovulation occurs between 24 and 32 h after GnRH). Pregnancy diagnoses were performed by ultrasound at 25 to 35 d post-AI. Pregnancy rates per AI were similar for the groups inseminated at 0, 8, 16, and 24 h and lower for the group inseminated at 32 h. A significant quadratic effect of treatment suggests that the middle time periods (8, 16, and 24 h) may produce the greatest pregnancy rate per AI. However, the group inseminated at 0 h had lowest pregnancy loss, and the group inseminated at 32 h tended to have the greatest pregnancy loss compared with that of the other groups. The calving rate was similar between the groups inseminated at 0, 8, 16, and 24 h and lower in the group inseminated at 32 h. The time of AI also appeared to affect gender of calf: cows bred at 0 and 32 h having a higher percentage of female offspring. In conclusion, there appears to be substantial flexibility in the time of AI after the second injection of GnRH, and lower reproductive rates were observed only when AI was after the time of ovulation.
(**Key words**: artificial insemination, synchronization of ovulation, pregnancy rate, gender ratio)

Abbreviation key: **PR/AI** = pregnancy rate per AI (the percentage of cows diagnosed pregnant by ultrasound examination at 25 to 35 d after a single AI).

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[1]Present address: Department of Animal Science, Anthony Hall, Michigan State University, East Lansing 48824.
[2]To whom correspondence should be addressed.

INTRODUCTION

When AI was being developed and validated, there were several studies (1, 3, 36, 37, 44) that were designed to determine the optimal time of AI in relation to estrus. The data suggested that optimal pregnancy rate per AI (**PR/AI**) would be achieved from midestrus until a few hours after the end of estrus. Since then, the recommended practice has been AI 12 h after the first observed estrus (a.m.-p.m. breeding). However, because of the variability of interval between the onset and the observation of estrus, it is difficult to define the ideal time of AI in relation to ovulation (8, 13, 36).

A protocol has been developed using GnRH and $PGF_{2\alpha}$ that synchronizes the time of ovulation within an 8-h period (24 to 32 h after the second injection of GnRH) with PR/AI similar to a.m.-p.m. breeding (5, 23, 24, 25, 30). This precise synchrony of ovulation allows for an effective test to determine the optimal time of AI in relation to ovulation.

MATERIALS AND METHODS

This trial was conducted on four large dairy farms located in three western states. Cows were milked three times daily, and the rolling herd averages ranged from 9980 to 11,800 kg of milk production per lactation. All four herds were owned by the same corporation (FMC Dairies, Inc., Salt Lake City, UT); hence, the management philosophies (e.g., nutrition, reproduction, and cow handling) were similar in each herd. A list of lactating dairy cows (n = 732) in all stages of lactation >50 d postpartum and known to be open, was faxed to our laboratory. On the basis of this list, cows were randomly assigned to five groups by stage of lactation and parity. The randomized lists were faxed back to the farm with specific instructions on the precise times of injections. College interns, hired for the summer, were responsible for performing the injections. All interns received hands-on training from a member of our laboratory prior to the start of the trial. Cow identification and time of injection were carefully recorded by the intern to ensure that cows were treated at the designated times. In addi-

tion, cow number and treatment time were written on each bottle of GnRH or $PGF_{2\alpha}$ by the intern immediately after each injection, and all empty bottles were mailed back to our laboratory for verification of correct treatment and time of injections.

All cows received the Ovsynch® protocol using GnRH (Cystorelin; Rhône-Merieux, Inc., Athens GA) and $PGF_{2\alpha}$ (Pharmacia & Upjohn Co., Kalamazoo, MI). Treatment groups were defined by the times that cows received a single AI after the second injection of GnRH. Cows in treatment groups 0, 8, 16, 24, and 32 h were inseminated once at 0, 8, 16, 24, or 32 h after the second injection of GnRH. Artificial inseminations were performed by herd personnel who normally performed this task, and service sires were chosen by the herd manager as part of the normal reproductive and genetic programs. Injections were staggered so that all cows, regardless of treatment, would be inseminated at the same time of day; the inseminator was blind to treatments. There were three breeding groups within each herd. The first breeding group received AI in mid-May, and the last breeding group received AI in late June 1994.

Pregnancy diagnosis was performed by ultrasound with a 7.5-MHz probe (Aloka 500 ultrasound machine; Corometrics Medical Systems, Wallingford, CT) at 25 to 35 d following AI. This diagnosis was confirmed by observation of a fetal heart beat. If the ultrasonographer was unsure of diagnosis, another pregnancy diagnosis was performed 3 wk later.

Calving dates were retrieved from farm computer records. Cows culled with no information on pregnancy status (n = 21) were excluded from pregnancy loss and calving rate calculations. The distribution of these cows was not different among treatment groups. If a cow that was initially diagnosed pregnant had a subsequent AI or was diagnosed as not pregnant prior to culling, then she was considered to have a pregnancy loss.

Data were analyzed using logistic regression. Covariates considered in the analysis of PR/AI were breeding date, farm, parity, DIM, and interactions of treatment by parity, treatment by DIM, DIM by parity, and treatment by DIM by parity. There were no significant interactions; therefore, PR/AI, rate of pregnancy loss, and calving rates were analyzed for linear and quadratic effects using maximum likelihood estimates with the logistic procedure of SAS (29). However, there was an interaction of DIM and parity. Chi-square was used to evaluate differences in PR/AI among stages of lactation within parities. Also, chi-square tests of independence were used to compare the PR/AI, calving rates, and pregnancy loss between all groups and between the groups inseminated at 0 or 32 h compared with the groups inseminated at 8, 16, or 24 h. The percentage of female calves was compared by the chi-square goodness-of-fit test to an expected population value of 45.8% obtained from a study (27) with 14,627 calves born to multiparous Holstein-Friesian cows (including single and twin births).

TABLE 1. Reproductive measures in lactating Holstein cows inseminated at specific times in relation to ovulation synchronized by an injection of GnRH.

	Time from second GnRH until AI					
	0 h	8 h	16 h	24 h	32 h	Total
Cows, no.	149	148	149	143	143	732
PR/AI,[a] %	37	41	45	41	32	39
Pregnancy loss,[b] %	9**	21	21	21	32**	22
Calving rate, %	31	31	33	29	20*	29
Twinning rate, %	0	6.5	0	2.5	3.5	2.4
Female:male, %	61:39[c]	45:55	54:46	54:46	65:35[c]	55:45

[a]Quadratic effect of treatment on pregnancy rate per AI (PR/AI) ($P < 0.01$).

[b]Linear effect of treatment on pregnancy loss ($P < 0.05$).

[c]Different ($P < 0.05$) when compared with expected female:male of 46:54.

*Different from other groups within row ($P < 0.05$).

**Different from other groups within row ($P < 0.10$).

RESULTS

The effects of treatment on PR/AI, calving rates, pregnancy loss, and gender of calves are shown in Table 1. The PR/AI was lower ($P < 0.05$) in the 32-h group than in the groups receiving AI at 0, 8, 16, and 24 h. There was a quadratic effect of treatment on PR/AI ($P < 0.05$), the lowest numerical values were found for cows bred at the earliest (0 h) and latest times (32 h), and the greatest value was found for cows bred at the middle time period (16 h). Pregnancy loss was lower ($P < 0.05$) for the 0-h group and tended ($P < 0.1$) to be greater for the 32-h group than for all other groups (Table 1). The effect ($P < 0.05$) of treatment on pregnancy loss was linear; the lowest losses occurred at the earliest time (0 h), and the greatest losses at the latest time (32 h). Calving rates were similar among the groups inseminated at 0, 8, 16, and 24 h and lower ($P < 0.05$) for cows in the 32-h group (Table 1). There was an effect of treatment on gender of calf (Table 1). There were more ($P < 0.05$) female than male calves born after AI at 0 and 32 h than would be expected (27). Gender ratio of calves born from AI at 8, 16, and 24 h were not different from expected values.

When stage of lactation and parity were analyzed (Table 2), PR/AI was greater ($P < 0.05$) for second

lactation cows than for cows in lactations 1 or ≥3. Also, PR/AI was lower ($P < 0.025$) in early postpartum cows (50 to 75 d) than in later postpartum cows (>76 d; Table 2), primarily because of lower ($P < 0.05$) PR/AI in early postpartum cows with ≥ 3 lactations.

TABLE 2. Pregnancy rates per AI for lactating dairy cows of different parities at various stages of lactation.[1]

Parity	Time postpartum 50–75 d		76–100 d		>100 d		Total	
	(%)	(n)	(%)	(n)	(%)	(n)	(%)	(n)
1	36	225	39	57	39	98	37	380
2	43[a]	104	70[b]	23	46	50	48[b]	177
≥3	26[c]	99	44	16	47	60	35	175
Total	36[c]	428	47	96	43	208	39	732

[a]Different from other parities ($P < 0.1$).

[b]Different from other parities ($P < 0.05$).

[c]Different from other stages of lactation within parity ($P < 0.05$).

[1]There were no interaction effects ($P > 0.1$) for treatment by parity or treatment by stage of lactation, so treatments were combined.

DISCUSSION

This study was designed to determine how time of AI affects reproductive performance of lactating dairy cows. The execution of this study was greatly facilitated by using a recently validated protocol that synchronizes the time of ovulation (5, 23, 24, 25, 30). This protocol synchronizes the growth of a new follicular wave (first injection of GnRH), synchronizes luteal function (first injection of GnRH and $PGF_{2\alpha}$ injection), and synchronizes the time of ovulation into an 8-h period (second injection of GnRH). The time of ovulation is between 24 to 32 h after the second injection of GnRH (24), which is similar to the time from onset of estrus until ovulation (42). Ovulation is synchronized in 87 to 100% of lactating dairy cows using this protocol (24, 39). The PR/AI after Ovsynch® is similar to (23, 25) or only slightly lower than (5, 33) AI after estrus in lactating dairy cows. Thus, use of the Ovsynch® protocol offers an ideal opportunity to reevaluate the optimal time of AI in lactating dairy cows. This study provides novel information because 1) a large number of cows (n = 732) were precisely synchronized; 2) predetermination of AI allowed cows to be randomly allocated to all treatments, allowed cows to be inseminated at the same time of day, and allowed the inseminator to be blind to treatment group; and 3) in addition to PR/AI evaluation, pregnancy loss, calving rate, and gender of calf were also evaluated.

A number of previous studies have evaluated the optimal time of AI. Early studies (1, 3, 36, 37, 44) evaluated pregnancy rates from AI performed at various times in relation to the onset of estrus. However, these studies suffered from low numbers of animals per treatment group, lack of statistical comparisons, variation in time of expressed estrus, and time from end of estrus until ovulation, or some combination of these factors. Since then, studies have shown similar PR/AI when cows were inseminated at the onset of estrus rather than at 12 h after the onset of estrus (8, 13) and when cows were inseminated once per day rather than by a.m.-p.m. breeding (10, 12). Correspondingly, breeding cows at the onset of estrus produced similar PR/AI as breeding at the onset of estrus and again 12 h later (41). Studies in New Zealand using fresh semen have indicated that the interval from AI to ovulation was a major factor contributing to fertility differences between sires (18). Fertility differences between sires were primarily observed with AI in early estrus and were not detectable for AI postestrus (18). In the current study, we found that PR/AI and calving rates were similar by chi-square analysis whether cows were bred at the time of the induced LH surge (0-h group, 24 to 32 h before ovulation), just prior to ovulation (24 h), or between these two times (8 and 16 h). As in past studies (3, 36, 37), PR/AI decreased when cows were bred after the time of ovulation. The significant quadratic effect of time of AI on PR/AI using logistic regression is similar to results of a previous study in beef cattle (26) and suggests there may be an ideal time of AI around 16 h after the second GnRH injection. Thus, there appears to be a substantial time period after the LH surge (~24 h) in which cows can be bred with acceptable PR/AI but an optimal time near 16 h after the LH surge seems likely from the data in this study.

The PR/AI were lower in this study than in earlier studies. There has been a trend for decreasing PR/AI in lactating dairy cows since the advent of AI. The PR/AI in lactating dairy cows was around 60% in the 1950s, was around 50% in the 1970s, and appears to be around 40% in the 1990s [(1, 23, 25, 32); current study]. A recent study of 19 well-monitored large New York herds found that PR/AI of lactating dairy cows averaged 40.9% (range, 32 to 50%) (6). The decreasing PR/AI is likely related to increased milk production per cow (19, 20); however, the precise physiological mechanisms are not well defined. In the current study, PR/AI are similar to the PR/AI in studies (23, 25) in which this protocol for synchronization of ovulation was compared with breeding to an estrus. In

those studies, there were no differences in PR/AI between the timed AI after synchronized ovulation compared with a.m.-p.m. AI after detection of estrus. In addition, the historical PR/AI on the farms used in this study were either similar to or lower than the PR/AI obtained in the study (data not shown). In the current study, the PR/AI of cows receiving AI in early lactation (50 to 75 d) was less than that of cows in later lactation (>75 d), similar to reported results of a previous study that used synchronization of ovulation (25). In the present study, this difference was primarily due to lower PR/AI in early postpartum cows in lactations ≥3. The reason why second lactation cows had greater PR/AI than cows in lactation 1 or ≥3 is unclear.

The 20% rate of pregnancy loss found in this study is similar to the 19% rate that was reported for primiparous and multiparous cows by Smith and Stevenson (31) when ultrasound was used to diagnose pregnancy at 28 to 30 d after AI. In contrast, Thurmond and Picanso (34) have reported pregnancy losses of 10.7% and Thurmond et al. (35) reported losses of 10.6% after routine pregnancy diagnosis by rectal palpation. In 1995, Forar et al. (9) reviewed 26 studies of endemic fetal loss and found a tremendous variation in fetal loss. Study populations, definitions of fetal loss, and procedural differences in designation and analysis of fetal losses appeared to be important sources of this variation. In our study, high pregnancy losses may reflect the high milk production in these herds and relatively early initial diagnosis of pregnancy. In a subsequent study, we have found a 10.5% loss of pregnancies between d 28 and 42 as determined by ultrasound (38). These high early pregnancy losses would not be expected to be detected when initial pregnancy diagnosis is by rectal palpation. Thus, the higher pregnancy loss in this study may reflect earlier pregnancy diagnosis although other differences in this study compared with past studies could also be important.

Interestingly, data from the current study indicate that the time of AI in relation to ovulation affected pregnancy losses. When AI occurred 24 to 32 h preovulation (0 h), the pregnancy loss was lower (9%) than that of other groups. These results are similar to those of another study (4) in which cows inseminated at first observed estrus had lower pregnancy loss between the first (30 to 40 d post-AI) and subsequent (60 to 70 d post-AI) diagnoses of pregnancy than did cows inseminated 12 h after first observed estrus. Overinterpretation of these data is discouraged because in a subsequent experiment we did not observe lower pregnancy losses in cows bred at 0 versus 24 h after the second GnRH injection (38).

The data from the current study may provide insight into the physiological mechanisms that affect fertilization of an oocyte and subsequent embryonic development. In the current study, when sperm were introduced into the uterus at any time from ~28 h prior to ovulation until just before ovulation, PR/AI were similar. Also, from results of the current study, the age of the oocyte at fertilization appears to affect PR/AI and pregnancy loss. The 32-h group had lower PR/AI and greater pregnancy loss. Similarly, embryonic loss by mares was greater when AI was performed after ovulation than when AI was prior to ovulation (46). About 8 h appear to be required for the sperm that are capable of fertilization to reach the isthmus portion of the oviduct (16). When this time is added to the time of AI after ovulation in the 32-h group (0 to 8 h after ovulation), the oocyte would have been between 8 to 16 h old at the earliest time of fertilization. The aging oocyte is likely to be the major contributor to the lower PR/AI and higher pregnancy loss in the group bred after ovulation. In contrast, the age of the oocyte should have been <8 h at fertilization in the other four groups and seems unlikely to be the major cause of reduced pregnancy loss in the 0 h group.

Sperm and oocyte age may also have an effect on sex ratio. More females resulted from AI at the same time as the LH surge (0 h group; 24 to 32 h before ovulation) and 32 h after the LH surge (32 h group; 0 to 8 h after ovulation) when compared with expected values of 45.8% female calves reported by Ryan and Boland (27). A skewed gender ratio (47% female) was also found using a data set of about 20,000 calvings from Holstein cattle in California (Steven Berry et al., 1997, personal communication). There are phenotypic differences between X- and Y-bearing sperm including plasma membrane surface charge, density, morphology (nucleus and head), and motility, and the degree of motility measured in effective velocity was greater in X-bearing sperm than in Y-bearing sperm (17, 45). Some studies of cattle (2, 7) indicated that there is no relationship between the time of insemination and sex ratio; others have suggested a relationship (21, 43). Studies of other species have reported substantial differences in sex ratio with regard to the relationship of time of insemination or intercourse and ovulation (11, 14, 15, 22, 40) and age of sperm at the time of insemination (28). From these studies, it appears that insemination around ≥1 d prior to ovulation results in more female offspring compared with the preponderance of males when insemination occurs around the time of ovula-

tion. Obviously, confirmation of this intriguing result in cattle requires further research.

In conclusion, the ability to synchronize precisely the time of ovulation in lactating dairy cows allowed calculation of how PR/AI, calving rate, pregnancy loss, and gender of calf were altered by varying the time from AI until ovulation. One unique optimal time for AI was not clearly demonstrated because similar PR/AI and calving rates were found with AI from 0 to 24 h after the second injection of GnRH. However, AI after ovulation resulted in lower PR/AI, lower calving rates, and greater pregnancy losses.

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Late Estrus or Metestrus Insemination After Estrual Inseminations Decreases Farrowing Rate and Litter Size in Swine[1]

K. J. Rozeboom*, M.H.T. Troedsson†, G. C. Shurson*, J. D. Hawton*, and B. G. Crabo*

Departments of *Animal Science and †Clinical and Population Sciences, University of Minnesota, St. Paul 55108

ABSTRACT: A study was conducted with 360 gilts and sows from four herds to determine whether fertility was affected when the final of multiple inseminations was performed in late estrus or metestrus (late insemination). Sows and gilts were inseminated at 24-h intervals immediately after the detection of estrus. After receiving a first insemination, females were paired according to parity and estrus status on d 2 of estrus. Each pair set was inseminated with extended semen from the same semen collection(s). Control females were inseminated once on d 1 if they were not in estrus on d 2 (n = 31) or on both days if they were in estrus on d 2 (n = 149). Late inseminated females in each pair were managed and inseminated in the same manner as control females and then inseminated again 24 h later regardless of estrus status. Overall reproductive performance was similar among the four herds. Late insemination caused a drop in farrowing rate in parity 1 and 2 females (23 and 22%; $P < .05$ and $P < .01$, respectively) and average litter size decreased by 1.1 pigs per litter ($P < .05$) regardless of parity. There were no differences in either litter size or farrowing rate between late inseminated females in estrus and those that were in metestrus at the time of their last insemination.

Key Words: Sow Reproduction, Artificial Insemination, Litter Size, Farrowing Rate

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Introduction

Artificial insemination of swine has become common in the United States (Flowers, 1995). Multiple inseminations (AI) during estrus is a standard procedure because double AI increase farrowing rate 8 to 12% and litter size by .2 pigs (Crabo and Dial, 1992), and a third AI reportedly offers a further slight advantage (Tilton and Cole, 1982; Reed et al., 1984; Hofmo, 1991). However, Flowers and Esbenshade (1993) reported that reproductive performance in gilts exhibiting estrus lasting less than 2 d could not be improved by increasing the mating frequency, and Dewey et al. (1995) found no association between the number of matings and litter size. Observations in our herds and by LaPierre (1994) suggest that three AI 24 h apart may be counterproductive to fertility and increase the incidence of vaginal discharge.

In swine, a large volume of semen is deposited directly into the uterus and induces an inflammation in the form of an influx of neutrophilic granulocytes beginning 2 h after insemination (Lovell and Getty, 1968; Pursel et al., 1978; Rodriguez-Martinez et al., 1990). Excess semen and inflammatory products need to be eliminated from the uterus to ensure an optimal embryonal environment before the embryos descend (Pope et al., 1990). The mechanism of uterine clearance is not known for sows, but it seems to be the result of increased myometrial activity in mares (Troedsson and Liu, 1991; Troedsson et al., 1993). Uterine motility in sows decreases dramatically in late estrus (Zerobin and Spörri, 1972; Bower et al., 1974), and this may impair uterine clearance. The objective of this study was to determine the effect of performing the last of multiple AI during late estrus or metestrus on farrowing rate and litter size in swine.

Materials and Methods

This experiment was conducted at four University of Minnesota experiment station farms with 360 Yorkshire × Landrace sows and gilts from May 1995 through June 1996. The distribution and reproductive performance of females among herds is shown in Table

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Table 1. Distribution of females, litter size, farrowing rate, and duration of estrus by herd[a]

Item	Herd 1	Herd 2	Herd 3	Herd 4
Herd size	72	100	110	125
Study females	68	84	104	104
Parity distribution (P0:P1:P2:P3)[b]	22:24:16:6	58:6:10:10	34:14:14:42	24:36:16:28
Litter size[c]				
Total born	11.1 ± .5	10.4 ± .6	11.1 ± .4	11.8 ± .4
Born alive	10.3 ± .5	9.6 ± .6	10.4 ± .4	10.2 ± .4
Farrowing rate[d]	.76 ± .02	.66 ± .02	.82 ± .01	.90 ± .01
Estrus duration[e]	69.9	62.5	61.6	64.2

[a]Interactions for herd × litter size, farrowing rate, and estrus duration were not significant ($P > .1$).
[b]Females grouped by parity; P0: gilt, P1: parity 1, P2: parity 2, P3: parity 3+.
[c]Least squares mean ± SE calculated from females farrowed during the study period, August 1995 to May 1996.
[d]Mean ± SE calculated from total females serviced during study, May 1995 to January 1996.
[e]Mean estrus duration calculated in hours for study females in each herd when estrus detection was performed once per day.

1. Females were monitored each morning for signs of estrus (standing heat reflex). Estrus was detected by applying back pressure to females while in the presence of a mature boar. Semen was collected from boars housed at each location and extended with VSP semen extender (IMV International, Minneapolis, MN). Semen from one or two boars was mixed with extender so that each insemination dose contained greater than 5×10^9 spermatozoa. Extended semen was used within 48 h of collection. Semen not used immediately was stored overnight at 18°C.

All females detected in estrus were immediately inseminated and considered for pairing (control vs treatment). On the 2nd d of estrus, females were placed in pairs according to estrus status, parity, and the order recorded on the breeding sheet on the 1st d of service. Females in each pair set were inseminated with the same batch of processed semen. Females of the same parity that were in estrus on d 2 formed a pair and were inseminated a second time. The latter female listed on the breeding record was then serviced a third time 24 h after the second insemination (treatment). One hundred forty-nine pairs of sows were in estrus on d 2, and one female in each pair received a third insemination. From the proportion of females that received a third insemination, 57 showed visual signs of estrus on d 3, 92 did not (metestrus). In pairs when neither female exhibited estrus on d 2, the latter female entered on the breeding record was serviced a second time 24 h after the first insemination (treatment). Thirty-one pairs of females did not exhibit signs of estrus on d 2, and one female in each pair received a second insemination (treatment). Females receiving their first insemination from the same semen collection that did not match with another female of the same parity group were excluded from this study. Females were divided into four groups based on their parity at the time of breeding: P0 (gilts), P1 (parity 1), P2 (parity 2), and P3 (all sows of parity 3 and older).

Data from 180 pairs of sows and gilts were analyzed. The following data were collected from each of the four herds: sow ID, parity, dates of insemination, service sire(s), farrowing date or date detected nonpregnant, number born, and number born alive. Estrus status for all females was recorded each day and mean estrus duration was calculated with the assumption that the average female was in estrus 12 h before it was detected or in metestrus 12 h after the last visual indication of estrus. Mummified fetuses were counted as dead pigs. Because each herd used different methods of pregnancy detection, 35-d conception rate was not used in the analysis of the results.

Litter size was analyzed using ANOVA in the GLM procedure of SAS (1994). The model included estrus response, parity, herd, pair nested within parity by herd, and treatment. Pair nested within parity by herd was used as the error term for heat response, parity, and herd. Interactions were initially included in the model but were removed because they were not significant. Mean comparisons were made with Fisher's protected LSD test.

Farrowing rate was analyzed using a GSK (Grizzle, Starmer, Koch) model (Agreste, 1990) for categorical data in the CATMOD procedure of SAS. The model included heat response, parity, herd, treatment, parity × treatment, and heat response × parity interactions. Contrasts were used to make treatment comparisons within each parity using the parity × treatment interaction term.

Results

Farrowing rate and estrus duration were similar among herds (Table 1), and significant differences in litter size by parity were only detected between gilts and parity 3 females ($P < .05$; Figure 1). A second or third insemination performed during late estrus or early metestrus decreased average litter size (Figure

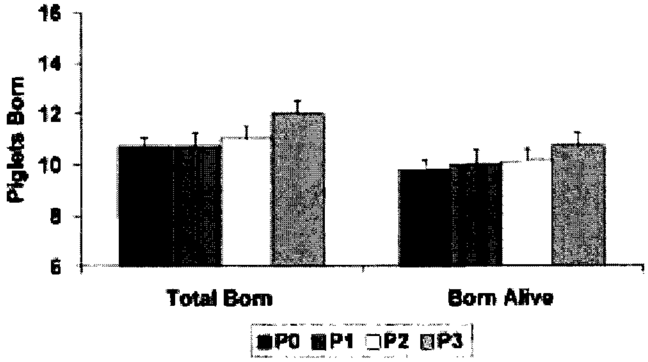

Figure 1. Comparison of litter size (mean ± SEM) among parity groups (P0: gilts; P1: parity 1 sows; P2: parity 2 sows; P3: parity 3+ sows). The P3 sows had more total pigs born compared to P0 gilts ($P < .05$). No other differences among parity groups were detected for total pigs born or born alive.

2). Control females had an average of 1.1 more total pigs born and 1.4 more pigs born alive per litter compared with late inseminated females ($P < .05$). Treatment interactions were significant for farrowing rate and parity. Parity 1 and parity 2 females showed the most marked difference in farrowing rate between control and treatment females ($P < .05$ and $P < .01$, respectively; Table 2), and although not significant, parity 0 and parity 3 control females showed a 19 and 2% advantage over late inseminated females. Farrowing rate (Table 2) and litter size (Figure 3) of late inseminated females were similar regardless of estrus status at the time of their last insemination.

Discussion

These results demonstrate that fertility and litter size decline when the last of multiple inseminations during one estrous cycle is performed during late estrus or metestrus. This observation was independent of whether the females were in estrus or not. The overall reproductive performance of the herds in this study was typical of well-managed commercial herds. Farrowing rate and litter size in this study ranks in the 70th percentile of the 1996 PigChamp® data summary (Thai, 1996). Thus, the results should be applicable under most farm conditions where AI is used.

The lowered fertility seen in this study shares apparent similarity to a previous study that showed when a single insemination is performed 0 to 24 h after ovulation (as measured by ultrasonography), farrowing rate and litter size are reduced compared to a single insemination 0 to 24 h before ovulation (Nissen et al., 1997). However, these results are not applicable to the present investigation in which previous insemination(s) were performed during the peak of estrus. Our results are in contrast to previous studies that suggested that a third insemination enhances reproductive performance (Tilton and Cole, 1982; Reed et al., 1984; Hofmo, 1991). However, this information was not given in the context of the insemination interval, which likely ranges between 12 and 24 h on most farms. Flowers and Esbenshade (1993) reported that increased frequencies of matings in females exhibiting estrus for 2 d improved reproductive performance, but the timing of inseminations was unimportant. Although not directly comparable to the study of Flowers and Esbenshade (1993), the present study clearly indicates that an insemination late in estrus may in fact counteract previous inseminations. It is important to emphasize that the results of our study do not imply that three inseminations or 24-h insemination intervals should be avoided, only that the last insemination should not be performed during late estrus or metestrus.

Recent studies have shown that the optimal insemination time for achieving high fertilization results is within 24 h before ovulation (Waberski et al., 1994; Kemp and Soede, 1996). Females that are observed in estrus for only 1 d are less receptive to the benefits of increased mating frequency (Flowers and Esbenshade, 1993) because ovulation likely occurs sometime within this 24-h period. Females with longer estrus periods benefit from increased mating frequency because there is a greater chance that a sufficient population of spermatozoa is present in the oviducts before ovulation. The results presented here emphasize that timing of the final insemination(s) is important so that the benefits of multiple inseminations are not voided.

The duration of estrus for all herds in this study was similar (63 h). The parity 0 females had the shortest estrus period (58 h), and parity 2 females

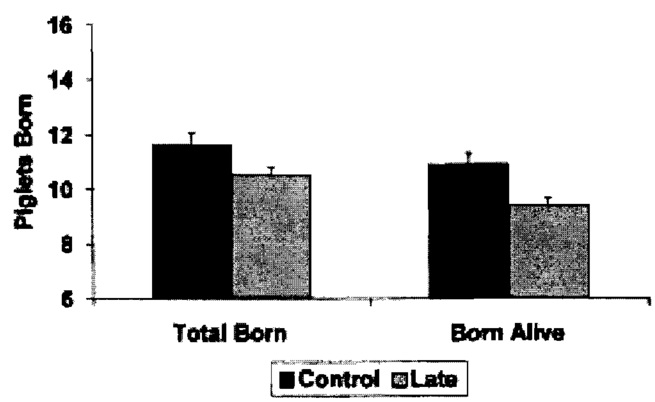

Figure 2. The effect of two or three inseminations (24-h interval) on total pigs born (mean ± SEM; 11.6 ± .4 vs 10.5 ± .3; $P < .05$) and born alive (10.9 ± .5 vs 9.4 ± .5; $P < .05$). Litter size was smaller for total pigs born and born alive when the last insemination was performed during late estrus or metestrus.

Table 2. Farrowing rate (mean ± SE) by parity for treatment and control females and farrowing rate according to estrus status of late inseminated females[a]

	Treatment[b]		Estrus status of late inseminated females[c]	
Parity	Control	Late	Estrus	Metestrus
0 (n = 138)	.78 ± .02 (n = 69)	.63 ± .03 (n = 69)	.92 ± .02 (n = 12)	.58 ± .03 (n = 57)
1 (n = 80)	.95 ± .01 (n = 40)	.73 ± .03 (n = 40)	.72 ± .05 (n = 18)	.72 ± .04 (n = 22)
2 (n = 56)	.89 ± .02 (n = 28)	.69 ± .04 (n = 28)	.44 ± .08 (n = 8)	.80 ± .04 (n = 20)
≥3 (n = 86)	.88 ± .02 (n = 43)	.86 ± .02 (n = 43)	.89 ± .02 (n = 19)	.83 ± .03 (n = 24)
Total	.86 ± .01 (n = 180)	.72 ± .02 (n = 180)	.76 ± .02 (n = 57)	.69 ± .02 (n = 123)

[a]Farrowing rate calculated from females serviced during study period: May 1995 to January 1996.
[b]Treatment × parity interaction ($P < .05$). Farrowing rate for late inseminated females was lower for P:1 and P:2 vs control females ($P < .05$ and $P < .01$, respectively).
[c]Farrowing rate × parity interaction ($P = .09$).

had the longest estrus period (69 h). Because the average estrus duration was not unusual (Weitze et al., 1994), the last insemination performed 72 h after the detection of estrus on d 3 was likely performed during late estrus or early metestrus. de Winter et al. (1992) showed that females inoculated with bacteria during late estrus and metestrus were more susceptible to uterine infection than sows inoculated during early to midestrus. These authors concluded that endometritis, vaginal discharge, and lower fertility are more common in herds in which sows are inseminated too late due to inaccurate estrus detection. Although this may be true, we are reluctant to ascribe the decreased reproductive performance in the present study entirely to an infection transmitted via the inseminate. A physiological postbreeding inflammation has been described in mares, in which a voluminous ejaculate also is deposited in the uterus, as a means to clean the uterus before embryos descend into it (Troedsson et al., 1995a,b). A similar mechanism may exist in pigs; neutrophilic granulocytes are known to enter the uterus following breeding (Lovell and Getty, 1968; Pursel et al., 1978; Rodriguez-Martinez et al., 1990; Bischof et al., 1994). All of these factors, in conjunction with an insufficient uterine contractility during late estrus and metestrus (Zerobin and Spörri, 1972; Bower et al., 1974), may have contributed to the lower fertility observed.

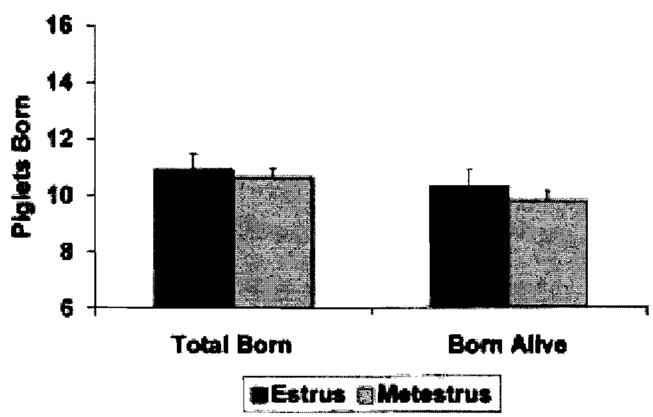

Figure 3. The number of total pigs born and born alive from females that were inseminated two or three times (24-h interval) when the last insemination was performed during late estrus or metestrus. No differences were detected between females that exhibited a standing heat reflex during the last insemination and females that did not exhibit a standing heat reflex during the last insemination on total pigs born (10.9 ± .5 vs 10.7 ± .3) or born alive (10.3 ± .5 vs 9.8 ± .3).

Implications

Even though multiple artificial inseminations during estrus have been shown to be beneficial to fertility in swine, performing the last insemination during late estrus or metestrus seems to reduce fertility.

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